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(54) Title: NOVEL APOPTOSIS-MODULATING PROTEINS, DNA ENCODING THE PROTEINS AND METHODS OF USE THEREOF

(57) Abstract

The present invention provides a novel family of apoptosis-modulating proteins. Nucleotide and amino acid residue sequences, derivatives thereof and methods of use thereof are also provided.

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NOVEL APOPTOSIS-MODULATING PROTEINS, DNA ENCODING THE PROTEINS AND METHODS OF USE THEREOF

This is a continuation-in-part of United States patent application Serial No. 08/320,157 which is a continuation-in-part of United States patent application Serial No. 08/160,067 filed November 30, 1993.

Field of the Invention

This invention relates to novel proteins with apoptosis-modulating activity, recombinant DNA encoding the proteins, compositions containing the proteins and methods of use thereof.

Background of the Invention

Apoptosis is a normal physiologic process that leads to individual cell death. This process of programmed cell death is involved in a variety of normal and pathogenic biological events and can be induced by a number of unrelated stimuli. Changes in the biological regulation of apoptosis also occur during aging and are responsible for many of the conditions and diseases related to aging. Recent studies of apoptosis have implied that a common metabolic pathway leading to cell death may be initiated by a wide variety of signals, including hormones, serum growth factor deprivation, chemotherapeutic agents, ionizing radiation and infection by human immunodeficiency virus (HIV). Wyllie (1980) Nature 284:555-556; Kanter et al. (1984) Biochem. Biophys. Res. Commun. 118:392-399; Duke and Cohen (1986) Lymphokine Res. 5:289-299; Tomei et al. (1988) Biochem. Biophys. Res. Commun. 155:324-331; Kruman et al. (1991) J. Cell. Physiol. 148:267-273; Ameisen and Capron (1991) Immunology Today 12:102; and Sheppard and Ascher (1992) J. AIDS 5:143. Agents that modulate the biological control of apoptosis thus have therapeutic utility in a wide variety of conditions.

Apoptotic cell death is characterized by cellular shrinkage, chromatin condensation, cytoplasmic blebbing, increased membrane permeability and internucleosomal DNA cleavage. Kerr et al. (1992) FASEB J. 6:2450; and Cohen and Duke (1992) Ann. Rev. Immunol. 10:267. The blebs, small, membrane-encapsulated spheres that pinch off of the surface of apoptotic cells, may continue to produce superoxide radicals which damage surrounding cell tissue and may be involved in inflammatory processes.

Bcl-2 was discovered at the common chromosomal translocation site t(14:18) in follicular lymphomas which results in aberrant over-expression of bcl-2. Tsujimoto et al. (1984) Science 226:1097-1099; and Cleary et al. (1986) Cell 47:19-28. The normal function of bcl-2 is the prevention of apoptosis; unregulated expression of bcl-2 in B cells is thought to lead to increased numbers of proliferating B cells which may be a critical factor in the development of lymphoma. McDonnell and Korsmeyer (1991) Nature 349:254-256; and, for review see, Edgington (1993) Bio/Tech. 11:787-792. *Bcl-2* is also capable of blocking γ irradiation-induced cell death. Sentman et al. (1991) Cell 67:879-888; and Strasser (1991) Cell 67:889-899. It is now known that bcl-2 inhibits most types of apoptotic cell death and is thought to function by regulating an antioxidant pathway at sites of free radical generation and Ca^{++} flux through the endoplasmic reticulum. Lan et al. (1984) Proc. Natl. Acad. Sci. 91:6569-6573; Hockenbery et al. (1993) Cell 75:241-251.

While apoptosis is a normal cellular event, it can also be induced by pathological conditions and a variety of injuries. Apoptosis is involved in a wide variety of conditions including, but not limited to, cardiovascular disease; cancer regression; immunoregulation; viral diseases; anemia; neurological disorders; gastrointestinal disorders, including but not limited to, diarrhea and dysentery; diabetes; hair loss; rejection of

organ transplants; prostate hypertrophy; obesity; ocular disorders; stress; and aging.

Bcl-2 belongs to a family of proteins some of which have been cloned and sequenced. Williams and Smith (1993) Cell 74:777-779. All references cited herein, both supra and infra, are hereby incorporated by reference herein.

Summary of the Invention

Substantially purified DNA encoding novel *Bcl-2* homologs, termed *Cdn-1*, *Cdn-2* and *Cdn-3* and derivatives thereof, as well as recombinant cells and transgenic animals expressing the *Cdn-1* and *Cdn-2* nucleotides are provided. The substantially purified Cdn-1 and Cdn-2 proteins and compositions thereof are also provided. Diagnostic and therapeutic methods utilizing the nucleotides and proteins are also provided. Methods of screening for pharmaceutical agents that stimulate, as well as pharmaceutical agents that modulate *Cdn-1* and *Cdn-2* expression and protein activity and interactions are also provided. Methods of screening for proteins that interact with Cdns are also provided.

Brief description of the Drawings

Figure 1 depicts the *Bcl-2* family PCR primers used to isolate the *Cdn-1* probes.

Figure 2 depicts the *Cdn-1* clones obtained by the methods described in Example 1.

Figure 3 depicts the nucleotide sequence of *Cdn-1* cDNA and encoded amino acid sequence of the Cdn-1 protein.

Figure 4 depicts the results of a Northern blot analysis of multiple tissues with probes specific for both *Bcl-2* and *Cdn-1*.

Figure 5 shows the sequence of the *Cdn-2* gene and flanking sequences and the corresponding predicted amino acid sequence of the Cdn-2 protein.

Figure 6 shows a comparison of the N-terminal amino acid sequences of Cdn-1, Cdn-2 and known Bcl-2 family members.

Figure 7 shows the nucleotide sequence of the *Cdn-3* gene and predicted amino acid sequence of the Cdn-3 protein.

Figure 8 shows the anti-apoptotic effects of *Cdn-1* and some of its derivatives in serum-deprivation induced apoptosis of WI-L2 transformants in 0.1% FBS.

Figure 9 (response of WIL-2 transformants to anti-Fas-Induced Apoptosis (50 ng/mL anti-FAS)) shows anti-apoptotic effects of *Cdn-1* and some of its derivatives in FAS-induced apoptosis of WI-L2 cells.

Figure 10 shows modulation of apoptosis by *Cdn-1* and *Cdn-2* in FL5.12 cells.

Figure 11 depicts the *Cdn-1* derivative proteins Δ1, Δ2 and Δ3. The N-terminal residues are indicated by the arrows. The remainder of the derivative proteins is the same as full-length Cdn-1.

Detailed Description of the Invention

The present invention encompasses substantially purified nucleotide sequences encoding the novel *Bcl-2* homologs, *Cdn-1* and *Cdn-2*; and the proteins encoded thereby; compositions comprising *Cdn-1* and *Cdn-2* nucleotides, and proteins and methods of use of thereof. Note that in copending United States patent application Serial No. 08/160,067, *Cdn-1* was termed cdi-1 and that in copending United States Patent application Serial No. 08/320,157 *Cdn* was termed cdn and Cdn was termed CDN; although the names have been changed, the nucleotide and amino acid sequences remain identical. The invention further includes recombinant cells and transgenic animals expressing the cloned *Cdn-1* or *Cdn-2* genes. The nucleotide and predicted amino acid residue sequences encoded by *Cdn-1* are shown in Figure 3; and those of *Cdn-2* are shown in Figure 5. It has now been found that the proteins encoded by the *Cdn* genes are capable of

modulating apoptosis. In an Epstein-Barr Virus (EBV) transformant lymphoblastoid cell line, *Cdn-1* was shown to decrease Fas-mediated apoptosis. In a mouse progenitor B cell line, FL5.12, expression of *Cdn-2* and a derivative of *Cdn-1* decrease IL-3-induced apoptosis whereas expression of *Cdn-1* slightly increased apoptosis. Thus, depending on the cell type, the derivative or type of *Cdn* expressed and the method of induction of apoptosis, apoptosis can be modulated in a highly specific manner by controlling the expression of *Cdns* and concentration of Cdns.

As used herein, "*Cdns*" or "*Cdn*" refers to the nucleic acid molecules (nucleotides) described herein (*Cdn-1*, *Cdn-2*, *Cdn-3* and derivatives thereof), "the Cdns" or "Cdn" refers to the proteins encoded thereby (Cdn-1, Cdn-2, Cdn-3 and derivatives thereof). The present invention encompasses *Cdn-1* and *Cdn-2* nucleotide sequences. The nucleotides include, but are not limited to, the *Cdn-1* cDNA, genome-derived DNA and synthetic or semi-synthetic nucleotides such as DNA, and RNA both coding and complementary to the coding region. The nucleotides may be complementary to the mRNA for at least a fragment of the Cdns and other nucleotides which can bind to either the DNA or mRNA encoding the Cdns. These complementary nucleotides include, but are not limited to, nucleotides capable of forming triple helices and antisense nucleotides. The complementary nucleotides may be expressed endogenously by one of the vectors described herein or may be added exogenously by methods known in the art of oligonucleotide therapy. Reed et al. (1990) Cancer Res. 50:6565-6570. The nucleotide sequence of the *Cdn-1* cDNA with the location of restriction endonuclease sites is shown in Figure 4. As described in the examples herein, *Cdn-1* mRNA has been detected in a variety of human organs and tissues by Northern blot analysis. These organs include liver; heart; skeletal muscle; lung; kidney; and pancreas as shown in Figure 3.

Similarly, *Cdn-2* cDNA, genomic DNA and synthetic or semi-synthetic nucleotides are additional embodiments of the present invention. The nucleotide sequence of the *Cdn-2* gene, along with the predicted amino acid sequence of *Cdn-2* protein and the locations of restriction endonuclease recognition sites, is given in Figure 5.

The examples presented herein indicate that *Cdn-1* is on human chromosome 6 and that *Cdn-2* is on human chromosome 20. There is also a member of the family *Cdn-3* which is on human chromosome 11. Fluorescence in situ hybridization (FISH) indicated an approximate location of *Cdn-1* to be at 6p21-23. It is possible that *Cdn-2* and *Cdn-3* are pseudogenes. While these may not be expressed endogenously, they are capable of being expressed from a recombinant vector providing the appropriate promoter sequences. Thus, both *Cdn-2* and *Cdn-3* nucleotide sequences are encompassed by the present invention as are recombinant constructs thereof and proteins encoded thereby.

Derivatives of the genes and proteins include any portion of the protein, or nucleotide sequence encoding the protein, which retains apoptosis modulating activity. Figure 11 depicts three such derivatives of *Cdn-1* which have been shown to retain apoptosis-modulating activity. The derivatives, *Cdn1-Δ1*, *Cdn1-Δ2* and *Cdn1-Δ3*, and the proteins encoded thereby are encompassed by the present invention.

The invention includes modifications to *Cdn* DNA sequences such as deletions, substitutions and additions particularly in the non-coding regions of genomic DNA. Such changes are useful to facilitate cloning and modify gene expression.

The invention further encompasses various substituted nucleotides. Substitutions can be made within the coding region that either do not alter the amino acid residues encoded or result in conservatively substituted amino acid residues. Nucleotide

substitutions that do not alter the amino acid residues encoded are useful for optimizing gene expression in different systems. Suitable substitutions are known to those of skill in the art and are made, for instance, to reflect preferred codon usage in the particular expression systems.

The invention encompasses functionally equivalent variants and derivatives of *Cdns* which may enhance, decrease or not significantly affect the properties of Cdns. For instance, changes in the DNA sequence that do not change the encoded amino acid sequence, as well as those that result in conservative substitutions of amino acid residues, one or a few amino acid deletions or additions, and substitution of amino acid residues by amino acid analogs are those which will not significantly affect its properties.

Amino acid residues which can be conservatively substituted for one another include but are not limited to: glycine/alanine; valine/isoleucine/leucine; asparagine/glutamine; aspartic acid/glutamic acid; serine/threonine; lysine/arginine; and phenylalanine/tyrosine. Any conservative amino acid substitution which does not significantly affect the properties of Cdns is encompassed by the present invention.

The invention further encompasses mutants of Cdns which, when expressed, interfere with the activity of endogenously expressed Cdns. Such mutants can be made by any method known in the art and screened for activity by their ability to affect activity of native Cdns.

Techniques for nucleic acid manipulation useful for the practice of the present invention are known in the art and described in a variety of references, including, but not limited to, <u>Molecular Cloning: A Laboratory Manual</u>, 2nd ed., vols. 1-3, eds. Sambrook et al. Cold Spring Harbor Laboratory Press (1989); and <u>Current Protocols in Molecular Biology</u>, eds. Ausubel et al.,

Greene Publishing and Wiley-Interscience: New York (1987) and periodic updates.

The invention further embodies a variety of DNA vectors having cloned therein the *Cdn* nucleotide sequences. Suitable vectors include any known in the art including, but not limited to, those for use in bacterial, mammalian, yeast and insect expression systems. Specific vectors are known in the art and need not be described in detail herein.

The vectors may also provide inducible promoters for expression of the *Cdn* nucleotide sequences. Inducible promoters are those which do not allow substantial constitutive expression of the gene but rather, permit expression only under certain circumstances. Such promoters may be induced by a variety of stimuli including, but not limited to, exposure of a cell containing the vector to a ligand, metal ion, other chemical or change in temperature. The promoters may also be cell-specific, that is, inducible only in a particular cell type and often only during a specific period of time. The promoter may further be cell cycle specific, that is, induced or inducible only during a particular stage in the cell cycle. The promoter may be both cell type specific and cell cycle specific. Any inducible or noninducible promoter known in the art is suitable for use in the present invention. Preferably, the promoter used is inducible.

The invention further includes a variety of expression systems transfected with the vectors. Suitable expression systems include, but are not limited to, bacterial, mammalian, yeast and insect. Specific expression systems and the use thereof are known in the art and are not described in detail herein.

The invention encompasses *ex vivo* transfection with *Cdn* nucleotide sequences, in which cells removed from animals including man are transfected with vectors containing *Cdn* nucleotides and reintroduced into animals.

Suitable transfected cells include individual cells or cells contained within whole tissues. In addition, *ex vivo* transfection can include the transfection of cells derived from an animal other than the animal or human subject into which the cells are ultimately introduced. Such grafts include, but are not limited to, allografts, xenografts, and fetal tissue transplantation. In addition, *in vivo* transfection such as by pulmonary administration of suitable vectors can be used.

Essentially any cell or tissue type can be treated in this manner. Suitable cells include, but are not limited to, cardiomyocytes and lymphocytes. For instance, lymphocytes, removed, transfected with the recombinant DNA and reintroduced into an HIV-positive patient may increase the half-life of the reintroduced T cells.

As an example, in treatment of HIV-infected patients by the above-described method, the white blood cells are removed from the patient and sorted to yield the $CD4^+$ cells. The $CD4^+$ cells are then transfected with a vector containing a *Cdn* nucleotide and reintroduced into the patient. Alternatively, the unsorted lymphocytes can be transfected with a recombinant vector having at least one *Cdn* nucleotide under the control of a cell-specific promoter such that only $CD4^+$ cells express the nucleotides. In this case, an ideal promoter would be the CD4 promoter; however, any suitable $CD4^+$ T cell-specific promoter can be used.

Further, the invention encompasses cells transfected *in vivo* by the vectors. Suitable methods of *in vivo* transfection are known in the art and include, but are not limited to, that described by Zhu et al. (1993) Science 261:209-211. *in vivo* transfection may be particularly useful as a prophylactic treatment for patients suffering from atherosclerosis. Modulation of the levels of Cdn could serve as prophylaxis for the apoptosis-associated reperfusion damage that results from

cerebral and myocardial infarctions. In these patients with a high risk of stroke and heart attack, the apoptosis and reperfusion damage associated with arterial obstruction could be prevented or at least mitigated.

Infarctions are caused by a sudden insufficiency of arterial or venous blood supply due to emboli, thrombi, or pressure that produces a macroscopic area of necrosis; the heart, brain, spleen, kidney, intestine, lung and testes are likely to be affected. Apoptosis occurs to tissues surrounding the infarct upon reperfusion of blood to the area; thus, modulation of Cdn levels, achieved by a biological modifier-induced change in endogenous production, by *in vivo* transfection or by anti-sense therapy, could be effective at reducing the severity of damage caused by heart attacks and stroke.

Transgenic animals containing the recombinant DNA vectors containing *Cdn* nucleotide sequences are also encompassed by the invention. Methods of making transgenic animals are known in the art and need not be described in detail herein. For a review of methods used to make transgenic animals, see, e.g., PCT publication no. WO 93/04169. Preferably, such animals express recombinant *Cdns* under control of a cell-specific and, even more preferably, a cell cycle-specific promoter.

In another embodiment, diagnostic methods are provided to detect the expression of *Cdns* either at the protein level or the mRNA level. Any antibody that specifically recognizes Cdns is suitable for use in Cdn diagnostics. Abnormal levels of Cdns are likely to be found in the tissues of patients with diseases associated with inappropriate apoptosis; diagnostic methods are therefore useful for detecting and monitoring biological conditions associated with such apoptosis defects. Detection methods are also useful for monitoring the success of Cdn-related therapies.

Purification or isolation of Cdns expressed either by the recombinant DNA or from biological sources such as

tissues can be accomplished by any method known in the art. Protein purification methods are known in the art. Generally, substantially purified proteins are those which are free of other, contaminating cellular substances, particularly proteins. Preferably, the purified Cdns are more than eighty percent pure and most preferably more than ninety-five percent pure. For clinical use as described below, the Cdns are preferably highly purified, at least about ninety-nine percent pure, and free of pyrogens and other contaminants.

Suitable methods of protein purification are known in the art and include, but are not limited to, affinity chromatography, immunoaffinity chromatography, size exclusion chromatography, HPLC and FPLC. Any purification scheme that does not result in substantial degradation of the protein is suitable for use in the present invention.

The invention also includes the substantially purified Cdns having the amino acid residue sequences depicted in Figures 3 and 5, respectively. The invention encompasses functionally equivalent variants of Cdns which do not significantly affect their properties and variants which retain the same overall amino acid sequence but which have enhanced or decreased activity. For instance, conservative substitutions of amino acid residues, one or a few amino acid deletions or additions, and substitution of amino acid residues by amino acid analogs are within the scope of the invention. Any conservative amino acid substitution which does not significantly affect the properties of Cdns is encompassed by the present invention.

Suitable antibodies are generated by using the Cdns as an antigen or, preferably, peptides encompassing the Cdn regions that lack substantial homology to the other gene products of the *Bcl-2* family. Methods of detecting proteins using antibodies and of generating antibodies

using proteins or synthetic peptides are known in the art and are not described in detail herein.

Cdn protein expression can also be monitored by measuring the level of *Cdn* mRNA. Any method for detecting specific mRNA species is suitable for use in this method. This is easily accomplished using the polymerase chain reaction (PCR). Preferably, the primers chosen for PCR correspond to the regions of the *Cdn* genes which lack substantial homology to other members of the Bcl gene family. Alternatively, Northern blots can be utilized to detect *Cdn* mRNA by using probes specific to Cdns. Methods of utilizing PCR and Northern blots are known in the art and are not described in detail herein.

Methods of treatment with *Cdns* also include modulating cellular expression of *Cdns* by increasing or decreasing levels of mRNA or protein. Suitable methods of modulating cellular expression of *Cdn* include, but are not limited to, increasing endogenous expression with biological modifiers; transfecting the cells with vectors encoding *Cdn* nucleotides so that either a *Cdn* gene is overexpressed or an anti-sense nucleotide is expressed; and expressing mutant Cdns which interfere with the interaction of endogenous Cdn with other proteins such as other members of the *Bcl-2* family. Cellular transfection is discussed above and is known in the art. Suitable indications for modulating endogenous levels of *Cdn* include, but are not limited to, malignancies and cardiac-specific expression. Cardiac specific expression is particularly suitable for use in indications including, but not limited to, patients susceptible to heart disease and in advance of cardiotoxic therapies including, but not limited to, chemotherapies such as adriamycin, so as to offer cardioprotection.

Modulating endogenous expression of *Cdns* can be accomplished by exposing the cells to biological modifiers that directly or indirectly change levels of Cdns either by modulating expression of *Cdns* or by

modulating degradation of *Cdn* mRNA. Suitable biological modifiers include, but are not limited to, molecules and other cells. Suitable molecules include, but are not limited to, drugs, cytokines, small molecules, hormones, combinations of interleukins, lectins and other stimulating agents e.g., PMA, LPS, bispecific antibodies and other agents which modify cellular functions or protein expression. Preferably, a suitable biological modifier is γIFN which increases Cdn expression levels in HT-29 cells. Further, biological modifiers include *Cdn* nucleotides which modify expression of endogenous *Cdn* and mutant Cdns which interfere with the activity of endogenous Cdns. Cells are exposed to such biological modifiers at physiologically effective concentrations, and the expression of *Cdns* is measured relative to a control not exposed to the biological modifiers. Those biological modifiers which change expression of *Cdns* relative to the control are selected for further study.

The methods of decreasing endogenous levels of *Cdns* include, but are not limited to, antisense nucleotide therapy and methods to deliver the sense on antisense construct and down-regulation of expression by biological modifiers. Antisense therapy is known in the art and its application will be apparent to one of skill in the art.

Screening for therapeutically effective biological modifiers is done either by exposing the cells to biological modifiers which may directly or indirectly modulate levels of Cdns either by changing expression or by altering the half-life of *Cdn* mRNA or Cdns. The biological modifiers may also interfere with Cdn-1 interactions with both other *Bcl-2* family members, and other gene products, e.g., proteases. Suitable biological modifiers include, but are not limited to, molecules and other cells. Suitable molecules include, but are not limited to, drugs, cytokines, small molecules, hormones, combinations of interleukins, lectins and other stimulating agents, e.g., PMA, LPS,

bispecific antibodies, *Cdn* nucleotides, Cdn mutants and other agents which modify cellular functions or protein expression. Cells are grown under conditions known to elicit expression of at least one *Cdn* (preferably *Cdn-1*), exposed to such biological modifiers at physiologically effective concentrations, and the expression of *Cdns* is measured relative to a control not exposed to biological modifiers. Those biological modifiers which modulate the expression of *Cdns* relative to a control are selected for further study. Cell viability is also monitored to ensure that altered *Cdn* expression is not due to cell death.

In determining the ability of biological modifiers to modulate (increase or decrease) *Cdn* expression, the levels of endogenous expression may be measured or the levels of recombinant fusion proteins under control of *Cdn*-specific promoter sequences may be measured. The fusion proteins are encoded by reporter genes.

Reporter genes are known in the art and include, but are not limited to chloramphenicol acetyl transferase (CAT) and β-galactosidase. Expression of *Cdn-1* and *Cdn-2* can be monitored as described above either by protein or mRNA levels. Expression of the reporter genes can be monitored by enzymatic assays, or antibody-based assays, like ELISAs and RIAs, also known in the art. Potential pharmaceutical agents can be any therapeutic agent or chemical known to the art, or any uncharacterized compounds derived from natural sources such as fungal broths and plant extracts. Preferably, suitable pharmaceutical agents are those lacking substantial cytotoxicity and carcinogenicity.

Suitable indications for modulating endogenous levels of *Cdns* are any in which *Cdn*-mediated apoptosis is involved. These include, but are not limited to, various types of malignancies and other disorders resulting in uncontrolled cell growth such as eczema, or deficiencies

in normal programmed cell death such as malignancies, including, but not limited to, B cell lymphomas.

The invention also encompasses therapeutic methods and compositions involving treatment of patients with biological modifiers to modulate expression of *Cdns*. Effective concentrations and dosage regimens may be empirically derived. Such derivations are within the skill of those in the art and depend on, for instance, age, weight and gender of the patient and type and severity of the disease. Alternatively, patients may be directly treated with either native or recombinant Cdns. The Cdns should be substantially pure and free of pyrogens. It is preferred that the recombinant Cdns be produced in a mammalian cell line so as to ensure proper glycosylation. Cdns may also be produced in an insect cell line.

For therapeutic compositions, a therapeutically effective amount of substantially pure Cdn or biological modifier or oligonucleotide that modulates the expression or activity thereof is suspended in a physiologically accepted buffer including, but not limited to, saline and phosphate buffered saline (PBS) and administered to the patient. Preferably administration is intravenous. Other methods of administration include but are not limited to, subcutaneous, intraperitoneal, gastrointestinal and directly to a specific organ, such as intracardiac, for instance, to treat cell death related to myocardial infarction.

Suitable buffers and methods of administration are known in the art. The effective concentration of a Cdn or biological modifier therefor will need to be determined empirically and will depend on the type and severity of the disease, disease progression and health of the patient. Such determinations are within the skill of one in the art.

Bcl-2 is thought to function in an antioxidant pathway. Veis et al. (1993) Cell 75:229-240. Therefore,

therapy involving *Cdns* is suitable for use in conditions in which superoxide is involved. Administration of modulators of *Cdn* expression results in an increased extracellular concentration of Cdns, which is thought to provide a method of directly inhibiting superoxide accumulation that may be produced by the blebs associated with apoptosis. The therapeutic method thus includes, but is not limited to, inhibiting superoxide mediated cell injury.

Suitable indications for therapeutic use of Cdns or biological modifier therefor are those involving free radical mediated cell death and include, but are not limited to, conditions previously thought to be treatable by superoxide dismutase. Such indications include, but are not limited to, HIV infection, autoimmune diseases, cardiomyopathies, neuronal disorders, hepatitis and other liver diseases, osteoporosis, and shock syndromes, including, but not limited to, septicemia.

Hybridization of cloned *Cdn* DNA to messenger mRNA from various regions of the brain indicated high levels of expression of *Cdn-1* in each of the regions studied (Figure 8). Therefore, neurological disorders are another area in which therapeutic applications of Cdns are indicated.

The invention further encompasses methods of assaying for interactions between Cdns and proteins which bind specifically to Cdns. The assays entail contacting purified Cdns with cell lysates containing a protein which may bind to Cdns under conditions sufficient for the protein to bind and assaying for the presence of the protein.

Typically the assay step involves contacting the protein with a specific binding partner such as an antibody which may be directly or indirectly labeled. Suitable assays include an ELISA that provides antibodies directed against the protein, in vitro translated Cdn and cell lysates containing the protein. Yeast genetic

systems such as the Matchmaker (Clontech) are also suitable for use in the assay.

The following examples are provided to illustrate but not limit the present invention. Unless otherwise specified, all cloning techniques were essentially as described by Sambrook et al. (1989) and all reagents were used according to the manufacturer's instructions.

Example 1

Identification and Cloning of *Cdn-1* cDNA

An amino acid sequence comparison of the six known *Bcl-2* family members (Figure 6) revealed two regions with considerable sequence identity, namely amino acids 144-150 and 191-199. In an attempt to identify new *Bcl-2* family members, degenerate PCR primers based on sequences in these regions were designed (Figure 1) and PCR was performed using human heart cDNA and human B lymphoblastoid cell line (WI-L2) cDNA. PCR was performed using the Hot Start/Ampliwax technique (Perkin Elmer Cetus). The final concentration of the PCR primers and the template cDNA were 4 μM and 0.1-0.2 ng/ml, respectively. The conditions for cDNA synthesis were identical to those for first strand cDNA synthesis of the cDNA library as described below. PCR was performed in a Perkin Elmer Cetus DNA Thermal Cycler according to the method described by Kiefer et al. (1991) Biochem. Biophys. Res. Commun. 176:219-225, except that the annealing and extension temperatures during the first 10 cycles were 36°C. Following PCR, samples were treated with 5 units of DNA polymerase I, Klenow fragment for 30 min at 37°C and then fractionated by electrophoresis on a 7% polyacrylamide, 1 X TBE (Tris/borate/EDTA) gel. DNA migrating between 170-210 base pairs was excised from the gel, passively eluted for 16 hours with gentle shaking in 10 mM Tris-HCl pH 7.5, 1 mM EDTA (TE), purified by passage over an Elutip-D column (Schleicher and Schuell), ligated to the pCR-Script vector (Stratagene) and

transformed into *Escherichia coli* strain XL1-Blue MRF (Stratagene). Plasmid DNA from transformants (white colonies) containing both the heart and WI-L2 PCR products was isolated using the Magic Miniprep DNA Purification System (Promega), and the DNA inserts were sequenced by the dideoxy chain termination method according to Sanger et al. (1977) Proc. Natl. Acad. Sci. USA 74:5463-5467 (USB, Sequenase version 2.0). DNA sequence analysis of the eleven heart PCR products revealed two sequences identical to *Bcl-x* (Boise et al. (1993) Cell 74:597-608) and ten other sequences unrelated to the *Bcl-2* family.

DNA sequence analyses of the eleven WI-L2 PCR products yielded one *Bcl-x* sequence, five sequences identical to another *Bcl-2* family member, bax (Oldvai et al. (1993) Cell 74:609-619), four unrelated sequences and one novel *Bcl-2* related sequence, termed *Cdn-1*. The unique *Cdn-1* amino acid sequence encoded by the PCR product is shown in Figure 6 from amino acid 151-190 (top row).

To isolate the *Cdn-1* cDNA, a human heart cDNA library (Clontech) and a WI-L2 cDNA library, constructed as described by Zapf et al. (1990) J. Biol. Chem. 265:14892-14898 were screened using the *Cdn-1* PCR DNA insert as a probe. The DNA was ^{32}P-labeled according to the method described by Feinberg and Vogelstein (1984) Anal. Biochem. 137:266-267 and used to screen 150,000 recombinant clones from both libraries according to the method described by Kiefer et al. (1991). Eight positive clones were obtained from the WI-L2 cDNA library. Four clones from the WI-L2 cDNA library and two from the heart cDNA library were further purified and plasmid DNA containing the cDNA inserts was excised from the λZAPII vector (Stratagene) (Figure 2). The two longest clones, W7 (2.1 kb) and W5 (2.0 kb) were sequenced and shown to contain the *Cdn-1* probe sequence, thus confirming their authenticity. Two clones from the heart cDNA library

were purified. The cDNA was subcloned into pBlsc and sequenced. The heart cDNAs also encoded *Cdn-1*.

The W7 DNA sequence along with the deduced amino acid residue sequence is shown in Figure 3. The deduced amino acid sequence of *Cdn-1* was also aligned for maximum sequence identity with the other *Bcl-2* family members and is shown in Figure 6. As can be seen, there is considerable sequence identity between *Cdn-1* and other family members between amino acids 100 and 200. Beyond this central region, sequence conservation falls off sharply. Like *Bcl-2*, *Cdn-1* appears to be an intracellular protein in that it does not contain either a hydrophobic signal peptide or N-linked glycosylation sites. *Cdn-1* does contain a hydrophobic C-terminus that is also observed with all *Bcl-2* family members except LMW5-HL, suggesting its site of anti-apoptotic activity, like that of *Bcl-2*, is localized to a membrane bound organelle such as the mitochondrial membrane, the endoplasmic reticulum or the nuclear membrane. Hockenbery et al. (1990); Chen-Levy et al. (1989) Mol. Cell. Biol. 9:701-710; Jacobsen et al. (1993) Nature 361:365-369; and Monighan et al. (1992) J. Histochem. Cytochem. 40:1819-1825.

Example 2

Northern Blot Analysis of cDNA Clones

Northern blot analysis was performed according to the method described by Lehrach et al. (1977) Biochem. 16:4743-4651 and Thomas (1980) Proc. Natl. Acad. Sci. USA 77:5201-5205. In addition, a human multiple tissue Northern blot was purchased from Clontech. The coding regions of *Bcl-2* and *Cdn-1* cDNAs were labeled by the random priming method described by Feinberg and Vogelstein (1984) Anal. Biochem. 137:266-267. Hybridization and washing conditions were performed according to the methods described by Kiefer et al. (1991). Specifically, Klenow-labeled fragments of *Bcl-2*

and *Cdn-1* clones were hybridized to a multiple human tissue Northern blot (Clontech 7760-1), at a final concentration of $1X10^6$ cpm/milliliter for each probe. The blot was washed at high stringency.

The results, presented in Figure 4 indicate that *Cdn-1* is expressed in all organs tested (heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas) whereas *Bcl-2* is not expressed or expressed at only low levels in heart, brain, lung, and liver. Thus, *Cdn-1* appears to be more widely expressed throughout human organs than *Bcl-2* and may be more important in regulating apoptosis in these tissues.

Example 3

<u>Expression of Recombinant *Cdn-1*</u>

In order to express recombinant *Cdn-1* in the baculovirus system, the *Cdn-1* cDNA generated in Example 1 was used to generate a novel *Cdn-1* vector, by a PCR methodology as described in Example 1, using primers from the 3′ and 5′ flanking regions of the gene which contain restriction sites to facilitate cloning. The plasmids were sequenced by the dideoxy terminator method (Sanger et al., 1977) using sequencing kits (USB, Sequenase version 2.0) and internal primers. This was to confirm that no mutations resulted from PCR.

A clone was used to generate recombinant viruses by *in vivo* homologous recombination between the overlapping sequences of the plasmid and AcNPV wild type baculovirus. After 48 hours post-transfection in insect *Spodoptera frugiperda* clone 9 (SF9) cells, the recombinant viruses were collected, identified by PCR and further purified. Standard procedures for selection, screening and propagation of recombinant baculovirus were performed (Invitrogen). The molecular mass, on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), of the protein produced in the baculovirus system was

compared with the predicted molecular mass of Cdn-1 according to the amino-acid sequence.

In addition, similar clones can be expressed preferably in a yeast intracellular expression system by any method known in the art, including the method described by Barr et al. (1992) Transgenesis ed. JAH Murray, (Wiley and Sons) pp. 55-79.

Example 4

Expression of *Cdn-1* in Mammalian Systems

The *Cdn-1* coding sequence was excised from the plasmid generated in Example 1, and introduced into plasmids pCEP7, pREP7 and pcDNA3 (Invitrogen) at compatible restriction enzyme sites. pCEP7 was generated by removing the RSV 3'-LTR of pREP7 with XbaI/Asp718, and substituting the CMV promoter from pCEP4 (Invitrogen). 25 μg of each *Cdn-1*-containing plasmid was electroporated into the B lymphoblastoid cell line WI-L2, and stable hygromycin resistant transformants or G418 resistant transformants (pcDNA3 constructs, Fig. 8) expressing *Cdn-1* were selected.

The coding region of *Cdns* can also be ligated into expression vectors capable of stably integrating into other cell types including, but not limited to, cardiomyocytes, neural cell lines such as GTI-7 and TNF sensitive cells such as the human colon adenocarcinoma cell line HT29 so as to provide a variety of assay systems to monitor the regulation of apoptosis by *Cdn-1*.

Example 5

Effect of the Anti-Apoptotic Activity of *Cdn-1* and its Derivatives in the Wild Type B Lymphoblastoid Cell Line WI-L2-729 HF2 and the Transformed Cell Expressing Excess *Cdn-1*

$2x10^5$ WI-L2, and WI-L2 cells transformed with a vector encoding *Cdn-1* as described in Example 4 were grown in RPMI supplemented with 10% fetal bovine serum

(FBS) for the anti-fas experiment or 0.1% FBS for serum deprivation experiments. In the case of the anti-fas experiment, after washing with fresh medium, the cells were suspended in RPMI supplemented with 10% FBS, exposed to anti-fas antibodies and the kinetics of cell death in response to an apoptosis inducing agent were analyzed by flow cytometry with FACScan. In the case of the serum deprivation experiment, the WI-L2 cells were resuspended in RPMI supplemented with 0.1% FBS and apoptosis was monitored according to the method described by Henderson et al. (1993) Proc. Natl. Acad. Sci. USA 90:8479-8483. Other methods of inducing apoptosis include, but are not limited to, oxygen deprivation in primary cardiac myocytes, NGF withdrawal, glutathione depletion in the neural cell line GTI-7 or TNF addition to the HT29 cell line. Apoptosis was assessed by measuring cell shrinkage and permeability to propidium iodide (PI) during their death. In addition, any other method of assessing apoptotic cell death may be used.

Figure 9 shows the anti-apoptotic response of various WI-L2 transformants to anti-Fas treatment. Figure 8 shows the anti-apoptotic response of various WI-L2 transformants to serum deprivation. In Figure 9, duplicate wells containing 3×10^5 cells were incubated with 50 ng/ml of the cytocidal anti-Fas antibody for 24 hours. Cell death was then analyzed by flow cytometry with FACScan. The proteins expressed from each construct are shown beneath the columns. Since many of the constructs are truncation or deletion variants, the exact amino acids expressed are also indicated. As can be seen, all of the transformants had some protective effect when compared to the control transformant containing the pREP7 vector alone. The most apoptosis-resistant transformant was the *Cdn-1Δ2* expressing cell line, in which over 90% of the cells survived anti-fas treatment. Significant protection was also observed in transformants expressing

full length *Cdn-1* (1-211) and *Cdn-1Δ1*, followed by *Bcl-2Δ* and *Bcl-2* expressing cell lines.

Cdn-1Δ1 and *Cdn-1Δ2* are lacking the nucleotides encoding the N-terminal 59 and 70 amino acids, respectively, of full length Cdn-1. The observation that expression of *Cdn-1Δ2* is more effective at blocking apoptosis than full length *Cdn-1* suggests that smaller, truncated Cdn-1 molecules may be potent therapeutics.

Example 6

Determination of other *Cdn* genes and Cloning of the *Cdn-2* Gene

Southern blot analyses of human genome DNA and a panel of human/rodent somatic cell DNAs indicated that at least 3 *Cdn* related genes and that they resided in chromosomes 6, 11 and 20. PCR/sequence analysis of the three hybrid DNAs showed that *Cdn-1* was on chromosome 6 and that two closely related sequences were on chromosome 20 (designated *Cdn-2*) and chromosome 11 (designated *Cdn-3*). We have cloned the *Cdn-2* and *Cdn-3* genes and sequenced them. Interestingly, both *Cdn-2* and *Cdn-3* do not contain introns and have all of the features of processed genes that have returned to the genome. *Cdn-3* has a nucleotide deletion, causing a frame shift and early termination and thus is probably a pseudogene. Both, however, have promoter elements [CCAAT, TATAAA boxes] but are probably not transcribed as determined by Northern blot analyses with *Cdn-2* and *Cdn-3* specified probes.

900,000 clones from a human placenta genomic library in the cosmid vector pWE15 (Stratagene, La Jolla, CA) were screened with a 950 bp *Bgl*II-*Hind*III cDNA probe containing the entire coding region of *Cdn-1*. The probe was ^{32}P-labeled according to the method of Feinberg and Vogelstein (1984) Anal. Biochem. 137:266-267. The library was processed and screened under high stringency hybridization and washing conditions as described by

Sambrook et al. (1989) Molecular Cloning, 2nd edition, Cold Spring Harbor Laboratory Press. Ten double positive clones were further purified by replating and screening as above. Plasmid DNA was purified using the Wizard Maxiprep DNA Purification System as described by the supplier (Promega Corp., Madison, WI) and analyzed by *Eco*RI restriction enzyme mapping and Southern blotting. The probe used for Southern blotting and hybridization conditions was the same as above.

The cosmid clones fell into two groups as judged by *Eco*RI restriction analysis and Southern blotting. Cosmid clones (*cos*) 1-4 and 7 displayed one distinct pattern of *Eco*RI generated DNA fragments and contained a single 6.5 kb hybridizing *Eco*RI DNA fragment. *Cos*2 and *Cos*9 fell into the second group that was characterized by a 5.5 kb hybridizing *Eco*RI DNA fragment. The 6.5 kb DNA fragment from *cos*2 and the 5.5 kb DNA fragment from *cos*9 were subcloned into pBluescript SK$^-$ (Stratagene, La Jolla, CA) using standard molecular biological techniques (Sambrook et al. as above). Plasmid DNA was isolated and the DNA inserts from two subclones, A4 (from *cos*2) and C5 (from *cos*9) were mapped with *Bam*HI, *Hind*III and *Eco*RI and analyzed by Southern blotting as described above. Smaller restriction fragments from both clones were subcloned into M13 sequencing vectors and the DNA sequence was determined.

The sequence of A4 contains an open reading frame that displays 97% amino acid sequence identity with *Cdn-1*. (Figure 5) The high degree of sequence identity of this gene with *Cdn-1* indicates that it is a new *Cdn-1* related gene and therefore will be called *Cdn-2*. A sequence comparison of the encoded *Cdn-2* protein and the other members of the *Bcl-2* family is shown in Figure 6. *Cdn-2* contains the conserved regions, BH1 and BH2, that are hallmarks of the *Bcl-2* family, and displays a lower overall sequence identity (~20-30%) to other members, which is also characteristic of the *Bcl-2* family. *Cdn-3*

has a frame shift resulting in a shorter, unrelated polypeptide and therefore does not contain the structural features of *Cdn-1*, *Cdn-2* or other *Bcl-2* family members.

Example 7
Chromosomal Localization of the *Cdn-1*, *Cdn-2* and *Cdn-3* Genes

Southern blot analysis of a panel of human/rodent somatic cell hybrid DNAs (Panel #2 DNA from the NIGMS, Camden, NJ) and fluorescent in situ hybridization (FISH) of metaphase chromosomes were used to map the *Cdn* genes to human chromosomes. For Southern blotting, 5 μg of hybrid panel DNA was digested with *Eco*RI or *Bam*HI/*Hind*III, fractionated on 0.8% or 1% agarose gels, transferred to nitrocellulose and hybridized with the *Cdn-1* probe. Hybridization and washing conditions were as described above. For FISH, the *Cdn-2* subclone, A4, was biotinylated using the Bionick Labeling System (Gibco BRL, Gaithersburg, MD) and hybridized to metaphase chromosomes from normal human fibroblasts according to the method described by Viegas-Pequignot in In Situ Hybridization, A Practical Approach, 1992, ed. D.G. Wilkinson, pp. 137-158, IRL Press, Oxford. Probe detection using FITC-conjugated avidin and biotinylated goat anti-avidin was according to the method described by Pinkel et al. (1988) Proc. Natl. Acad. Sci. USA 85:9138-9142.

Southern blot analysis showed three hybridizing *Eco*RI bands in the human DNA control that were approximately 12 kb, 11 kb and 5.5 kb in length. Analysis of the somatic cell hybrid DNA indicated that the 12 kb band was in two different samples, NA10629, which contained only human chromosome 6, and NA07299, which contained both human chromosomes 1 and X and, importantly, a portion of chromosome 6 telomeric to p21. The 11 kb band was in NA13140, which contains human chromosome 20. The 5.5 kb hybridizing band was found

only in sample NA10927A, which contained human chromosome 11. PCR/DNA sequencing analysis of these hybrid DNA samples using primers for *Cdn-1*, *Cdn-2* or *Cdn-3* showed *Cdn-1* sequences in NA10629 (the chromosome 6-containing hybrid DNA) and NA07299 (the chromosome 1, X and 6pter >p21-containing hybrid DNA), indicating that the *Cdn-1* gene resides on chromosome 6, telomeric to p21. *Cdn-2* sequences were found in NA13140, indicating the *Cdn-2* gene resides on chromosome 20, and *Cdn-3* sequences were found in NA10927A, indicating the *Cdn-3* gene resides on chromosome 11.

Example 8

Modulation of apoptosis by <u>*Cdn-1* and *Cdn-2* in FL5.12 cells</u>

FL5.12 is an IL-3-dependent lymphoid progenitor cell line (McKearn et al. (1985) <u>Proc. Natl. Acad. Sci USA</u> <u>82</u>:7414-7418) that has been shown to undergo apoptosis following withdrawal of IL-3 but is protected from cell death by overexpression of *Bcl-2*. Nunez et al. (1990) <u>J. Immunol.</u> <u>144</u>:3602-3610; and Hockenbery et al. (1990) <u>Nature</u> <u>348</u>:334-336. To assess the ability of *Cdn-1* and *Cdn-2* to modulate apoptosis, cDNAs encoding *Cdn-1*, *Cdn-2*, two truncated forms of *Cdn-1* (described below) and *Bcl-2* were ligated into the mammalian expression vector, pcDNA3 (Invitrogen, San Diego, CA) and stably introduced into the mouse progenitor B lymphocyte cell line FL5.12 by electroporation and selection in media containing the antibiotic G418. Assays were then performed on bulk transformants as described below.

The effects of the overexpressed genes on FL5.12 cell viability were examined at various times following withdrawal of IL-3 and are shown in Figure 10. Cell viability was assessed by propidium iodide (PI) exclusion on a flow cytometer (Becton Dickinson FACScan). *Bcl-2* expression protected the cells significantly from cell death while *Cdn-1* appeared to enhance cell death when

compared to the vector control. *Cdn-2* expression conferred a low level of protection from cell death at earlier times but was insignificant at later time points. Interestingly, *Cdn-1Δ2* gave a moderate level of protection against cell death. *Cdn-1-112*, a molecule that contains the N-terminal 112 amino acids of *Cdn-1*, also appeared to partially protect the FL5.12 cells although at lower levels than *Bcl-2*.

As shown in Example 7, expression of *Cdn-1* and *Cdn-1Δ2* in WI-L2 cells resulted in increased cell survival in response to anti-Fas-mediated apoptosis and serum withdrawal. Taken together, these data suggest that the various *Cdn* molecules are capable of modulating apoptosis in a positive or negative manner, depending on the cell type and apoptotic stimuli. Thus, they are effective in preventing cell death such as in the post-ischemic reperfusion tissue damage in the heart or in inducing cell death in cells that have escaped apoptotic control, as is the case in various cancers.

Example 9

<u>IFN-γ induces *Cdn-1* mRNA expression in HT-29 cells</u>

It has been shown that the human colon carcinoma cell line HT-29 is sensitive to the cytotoxic effect of anti-Fas antibody or TNF only after treatment with IFN-γ. Yonehara et al. (1989) <u>J. Exp. Med.</u> <u>169</u>: 1747-1756. These IFN-γ treated cells also show enhanced apoptosis following serum deviation or cycloheximide treatment. This induced sensitivity of HT-29 cells to apoptotic stimuli may be partly due to the concomitant upregulation of the TNF receptor and Fas antigen that is seen following IFN-γ treatment. Yonehara et al. (1989). However, the increased cell death seen following serum deprivation or cycloheximide treatment suggests that other apoptotic mechanisms may be induced by IFN-γ.

Modulation of the levels of *Bcl-2* family members by IFN-γ is another possible mechanism for the induced

sensitivity of HT-29 cells to apoptotic stimuli. An increase in *Cdn-1*, Bax or *Bcl-x*$_s$ expression and/or a decrease in *Bcl-2* or *Bcl-x*$_L$ expression could result in enhanced sensitivity of the cells to cytocidal agents. To test this possibility, the mRNA levels of *Bcl-2* family members in untreated and IFN-γ treated HT-29 cells were examined by Northern blot analysis using the methods and conditions described in Example 2. *Cdn-1* mRNA levels were increased approximately 10X following IFN-γ induction whereas Bax and *Bcl-x* mRNA levels remained unchanged. *Bcl-2* mRNA was below detectable levels in both untreated and IFN-γ treated cells. It remained possible that the ratio of *Bcl-x*$_s$ to *Bcl-x*$_L$ transcripts could have increased upon IFN-γ treatment but would not be detected by Northern blot analysis due to the small difference in size between the transcripts. This was the case with unstimulated versus PMA plus ionomycin stimulated thymocytes as determined by Boise et al. (1993) Cell 74:597-608. Using semiquantitative PCR, they showed that the ratio of *Bcl-x*$_s$ to *Bcl-x*$_L$ increased following stimulation. Using similar PCR techniques it was demonstrated that the ratio of *Bcl-x*$_s$ to *Bcl-x*$_L$ mRNA remained unchanged following IFN-γ treatment of HT-29 cells and that the predominant transcript was *Bcl-x*$_L$ (>90%).

Thus there is a positive correlation between the upregulation of *Cdn-1* transcripts and the HT-29 tumor cells line following IFN-γ treatment and increased susceptibility to cell death. These results indicate that there are positive modulators of *Cdn-1* in tumor cells and that they may be useful in treating some tumors when co-administered with appropriate apoptosis inducing agents.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and

modifications may be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

We claim:

1. A composition comprising a substantially purified nucleotide sequence encoding a *Cdn*.

2. The composition according to claim 1 wherein the nucleotide sequence is derived from genomic DNA.

3. The composition according to claim 1 wherein the *Cdn* is *Cdn-1*.

4. The composition according to claim 3 having the nucleotide sequence depicted in Figure 3.

5. The composition according to claim 1 wherein the *Cdn* is *Cdn-2*.

6. The composition according to claim 5 having the nucleotide sequence depicted in Figure 5.

7. A composition comprising a recombinant DNA vector encoding a Cdn.

8. The composition according to claim 7 wherein the Cdn is Cdn-1.

9. The composition according to claim 8 wherein the nucleotide sequence is depicted in Figure 3.

10. The composition according to claim 7 wherein the Cdn is Cdn-2.

11. The composition according to claim 10 wherein the nucleotide sequence is depicted in Figure 5.

12. The recombinant DNA vector according to claim 7 wherein expression of the sequence encoding the *Cdn* under control of an inducible promoter.

13. A composition comprising a cell transfected with a recombinant DNA vector encoding a Cdn.

14. The composition according to claim 13 wherein the Cdn is Cdn-1.

15. The composition according to claim 14 wherein the nucleotide sequence is depicted in Figure 3.

16. The composition according to claim 13 wherein the Cdn is Cdn-2.

17. The composition according to claim 16 wherein the nucleotide sequence is depicted in Figure 5.

18. A transgenic animal comprising a recombinant DNA vector encoding a Cdn.

19. The transgenic animal according to claim 18 wherein the Cdn is Cdn-1.

20. The transgenic animal according to claim 19 wherein the *Cdn* nucleotide sequence is depicted in Figure 3.

21. The transgenic animal according to claim 18 wherein the Cdn is Cdn-2.

22. The transgenic animal according to claim 21 wherein the *Cdn* nucleotide sequence is depicted in Figure 5.

23. A composition comprising a substantially purified Cdn protein.

24. The composition according to claim 23 wherein the protein is Cdn-1.

25. The composition according to claim 24 wherein the nucleotide sequence is depicted in Figure 3.

26. The composition according to claim 23 wherein the Cdn is Cdn-2.

27. The composition according to claim 26 wherein the nucleotide sequence is depicted in Figure 5.

28. The composition according to claim 23 wherein the proteins are expressed by recombinant DNA.

29. The composition according to claim 23 wherein the proteins are native proteins.

30. A composition comprising the proteins according to claim 23 and a pharmaceutically acceptable buffer.

31. The composition according to claim 30 wherein the proteins are present in therapeutically effective amounts.

32. A composition comprising a monoclonal or polyclonal antibody which recognizes a Cdn but is substantially unreactive with other members of the Bcl family.

33. A method of detecting the presence of a Cdn protein in a biological sample comprising the steps of:

a) obtaining a cell sample;

b) lysing or permeabilizing the cells to antibodies;

c) adding anti-*Cdns*-specific antibodies to the cell sample;

d) maintaining the cell sample under conditions that allow the antibodies to complex with the cdn; and

e) detecting the antibody-*cdn* complexes formed.

34. The method according to claim 33 wherein the Cdn is Cdn-1.

35. The method according to claim 34 wherein the nucleotide sequence is depicted in Figure 3.

36. The method according to claim 33 wherein the Cdn is Cdn-2.

37. The method according to claim 36 wherein the nucleotide sequence is depicted in Figure 5.

38. The method according to claim 32 wherein the cell sample comprises T cells.

39. A method for detecting the expression of a *Cdn* gene in a biological sample comprising the steps of identifying the presence of RNA encoding the cdn.

40. The method according to claim 39 wherein the method for identifying the *Cdn-1* or *Cdn-2* mRNA is Northern blotting.

41. A method identifying *Cdn* mRNA comprising the steps of:

a) obtaining a cell sample;

b) obtaining RNA from the cell sample;

c) performing a polymerase chain reaction on the RNA using primers corresponding to unique regions of the *Cdn;* and

d) detecting the presence of products of the polymerase chain reaction.

42. A method of modulating apoptosis-induced cell death comprising modulating the endogenous levels of a Cdn.

43. The method according to claim 40 wherein the Cdn is Cdn-1.

44. The method according to claim 43 wherein the nucleotide sequence is depicted in Figure 3.

45. The method according to claim 42 wherein the Cdn is Cdn-2.

46 . The method according to claim 45 wherein the nucleotide sequence is depicted in Figure 5.

47. The method according to claim 41 wherein the Cdn is increased by modulating expression of an endogenous cdn gene.

48. The method according to claim 46 wherein the *Cdn* gene expressed is encoded by a recombinant gene.

49. The method according to claim 48 wherein expression of the gene is under the control of an inducible promoter.

50. The method according to claim 49 wherein the cells and transfected *ex vivo* and further comprising the steps of reintroducing the transfected cells into the animal.

51. The method according to claim 50 wherein the cells are T lymphocytes.

52. The method according to claim 49 wherein the recombinant gene is transfected into cells *in vivo*.

53. A method of treating apoptosis in a patient in need thereof comprising administering a therapeutically effective amount of Cdn.

54. The method according to claim 53 wherein the Cdn is Cdn-1.

55. The method according to claim 54 wherein the nucleotide sequence is depicted in Figure 3.

56. The method according to claim 53 wherein the Cdn is Cdn-2.

57. The method according to claim 56 wherein the nucleotide sequence is depicted in Figure 5.

58. The method according to claim 53 wherein the Cdn is administered for any indication for which superoxide dismutase has been indicated.

59. A method of assaying for interactions between Cdns and proteins which bind specifically to Cdns comprising the steps of contacting purified Cdn with cell lysates containing a protein which may bind to the Cdn under conditions which allow binding of the protein and the Cdn;

isolating the Cdn;

contacting the isolated Cdn with a binding partner specific for the protein under conditions which allow binding of the binding partner and the Cdn; and

measuring the amount of binding partner bound to the protein.

60. The method according to claim 59 wherein the cell lysate is a yeast lysate.

61. The method according to claim 59 wherein the binding partner is an antibody.

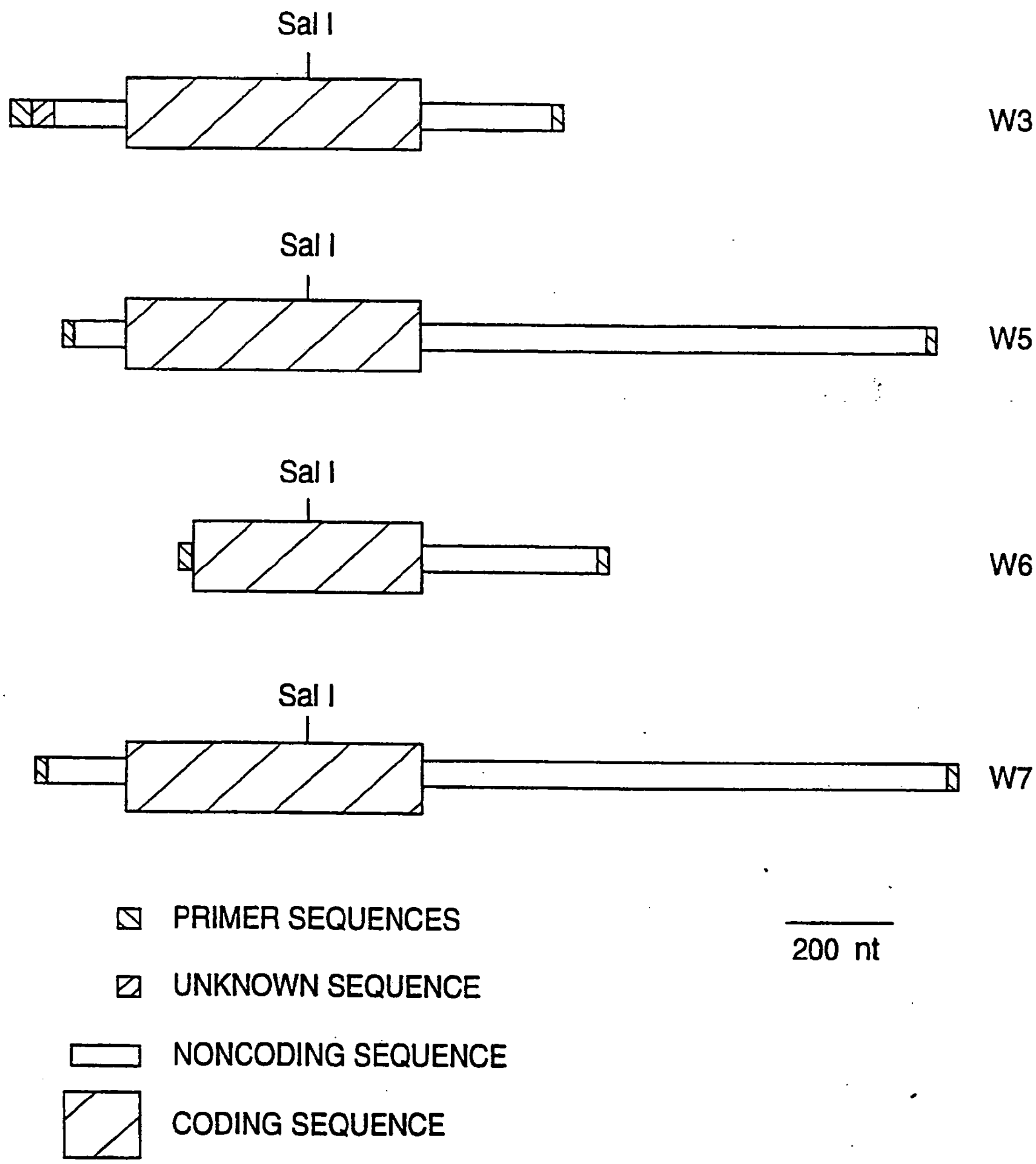
5'- AGATCTGAATTC AA(C/T) TGG GGI (C/A)GI (A/G)TX GTX GC -3'

Bclx 1-32

5'- AGATCTAAGCTT GTC CCA ICC ICC XTG XTC (C/T)TG (A/T/G)AT CCA -3'

Bclx 2-39

FIG. 1



Sal I
W3
Sal I
W5
Sal I
W6
Sal I
W7
PRIMER SEQUENCES
UNKNOWN SEQUENCE
NONCODING SEQUENCE
CODING SEQUENCE
200 nt

FIG. 2

```
                                        >Aha2
                                            |                                     80
          *         *         *         *   |     *         *         *         *
GGACAAGTAA AGGCTACATC CAGATGCCGG GAATGCACTG ACGCCCATTC CTGGAAACTG GGCTCCCACT CAGCCCCTGG

                                                       >Sma1      >BamH1
                                                        |           |            160
          *         *         *         *         *     |   *       |    *         *
GAGCAGCAGC CGCCAGCCCC TCGGACCTCC ATCTCCACCC TGCTGAGCCA CCCGGGTTGG GCCAGGATCC CGGCAGGCTG

          *         *         *          *         *         *
ATCCCGTCCT CCACTGAGAC CTGAAAA ATG GCT TCG GGG CAA GGC CCA GGT CCT CCC AGG CAG GAG TGC
                               M   A   S   G   Q   G   P   G   P   P   R   Q   E   C>

         240
*         *         *         *         *         *         *
GGA GAG CCT GCC CTG CCC TCT GCT TCT GAG GAG CAG GTA GCC CAG GAC ACA GAG GAG GTT TTC CGC
 G   E   P   A   L   P   S   A   S   E   E   Q   V   A   Q   D   T   E   E   V   F   R>

                    320
    *         *         *         *         *         *         *
AGC TAC GTT TTT TAC CGC CAT CAG CAG GAA CAG GAG GCT GAA GGG GTG GCT GCC CCT GCC GAC CCA
 S   Y   V   F   Y   R   H   Q   Q   E   Q   E   A   E   G   V   A   A   P   A   D   P>

                                            >Nco1
                                              |  400
        *         *         *                 |   *         *         *
GAG ATG GTC ACC TTA CCT CTG CAA CCT AGC AGC ACC ATG GGG CAG GTG GGA CGG CAG CTC GCC ATC
 E   M   V   T   L   P   L   Q   P   S   S   T   M   G   Q   V   G   R   Q   L   A   I>

                                                                              >Pst1
                                                                    480         |
  *         *         *         *         *         *         *         *       |*
ATC GGG GAC GAC ATC AAC CGA CGC TAT GAC TCA GAG TTC CAG ACC ATG TTG CAG CAC CTG CAG CCC
 I   G   D   D   I   N   R   R   Y   D   S   E   F   Q   T   M   L   Q   H   L   Q   P>

                   >Sca1_
                        |
        *         *     |   *         *         *         *         *
ACG GCA GAG AAT GCC TAT GAG TAC TTC ACC AAG ATT GCC ACC AGC CTG TTT GAG AGT GGC ATC AAT
 T   A   E   N   A   Y   E   Y   F   T   K   I   A   T   S   L   F   E   S   G   I   N>

560
*         *         *         *         *         *         *
TGG GGC CGT GTG GTG GCT CTT CTG GGC TTC GGC TAC CGT CTG GCC CTA CAC GTC TAC CAG CAT GGC
 W   G   R   V   V   A   L   L   G   F   G   Y   R   L   A   L   H   V   Y   Q   H   G>
```

FIG. 3A SUBSTITUTE SHEET (RULE 26)

```
                                       >Sal1
                     640                 |
       *              *         *        *|          *          *          *
CTG ACT GGC TTC CTA GGC CAG GTG ACC CGC TTC GTG GTC GAC TTC ATG CTG CAT CAC TGC ATT GCC
 L   T   G   F   L   G   Q   V   T   R   F   V   V   D   F   M   L   H   H   C   I   A>

                                 720
         *          *             *          *          *          *
CGG TGG ATT GCA CAG AGG GGT GGC TGG GTG GCA GCC CTG AAC TTG GGC AAT GGT CCC ATC CTG AAC
 R   W   I   A   Q   R   G   G   W   V   A   A   L   N   L   G   N   G   P   I   L   N>

                                                       800
 *          *          *          *          *          *          *
GTG CTG GTG GTT CTG GGT GTG GTT CTG TTG GGC CAG TTT GTG GTA CGA AGA TTC TTC AAA TCA TGA
 V   L   V   V   L   G   V   V   L   L   G   Q   F   V   V   R   R   F   F   K   S   *>

                                        >Af12
                                          |        880
     *          *          *          *   |*          *          *          *
CTCCCAA GGGTGCCCTT TGGGTCCCGG TTCAGACCCC TGCCTGGACT TAAGCGAAGT CTTTGCCTTC TCTGTTCCCT

                          >Hind3
                            |                                  960
         *          *       |  *          *          *          *          *          *
TGCAGGGTCC CCCCTCAAGA GTACAGAAGC TTTAGCAAGT GTGCACTCCA GCTTCGGAGG CCCTGCGTGG GGGCCAGTCA

   >Pst1                                      >Apa1
    |                                            |    1040
    | *          *          *          *          *|          *          *          *
GGCTGCAGAG GCACCTCAAC ATTGCATGGT GCTAGTGCCC TCTCTCTGGG CCCAGGGCTG TGGCCGTCTC CTCCCTCAGC

                                                      1120
          *          *          *          *          *          *          *          *
TCTCTGGGAC CTCCTTAGCC CTGTCTGCTA GGCGCTGGGG AGACTGATAA CTTGGGGAGG CAAGAGACTG GGAGCCACTT

                                                      1200
          *          *          *          *          *          *          *          *
CTCCCCAGAA AGTGTTTAAC GGTTTTAGCT TTTTATAATA CCCTTGTGAG AGCCCATTCC CACCATTCTA CCTGAGGCCA

>Aha2
 |                                                    1280
 |        *          *          *          *          *          *          *          *
GGACGTCTGG GGTGTGGGGA TTGGTGGGTC TATGTTCCCC AGGATTCAGC TATTCTGGAA GATCAGCACC CTAAGAGATG

                                                      1360
          *          *          *          *          *          *          *          *
GGACTAGGAC CTGAGCCTGG TCCTGGCCGT CCCTAAGCAT GTGTCCCAGG AGCAGGACCT ACTAGGAGAG GGGGGCCAAG
```

```
                                                            1440
         *         *         *         *         *         *         *         *
GTCCTGCTCA ACTCTACCCC TGCTCCCATT CCTCCCTCCG GCCATACTGC CTTTGCAGTT GGACTCTCAG GGATTCTGGG

                                                            1520
         *         *         *         *         *         *         *         *
CTTGGGGTGT GGGGTGGGGT GGAGTCGCAG ACCAGAGCTG TCTGAACTCA CGTGTCAGAA GCCTCCAAGC CTGCCTCCCA

                                                            1600
         *         *         *         *         *         *         *         *
AGGTCCTCTC AGTTCTCTCC CTTCCTCTCT CCTTATAGAC ACTTGCTCCC AACCCATTCA CTACAGGTGA AGGCTCTCAC

                                                            1680
         *         *         *         *         *         *         *         *
CCATCCCTGG GGGCCTTGGG TGAGTGGCCT GCTAAGGCTC CTCCTTGCCC AGACTACAGG GCTTAGGACT TGGTTTGTTA

                                                            1760
         *         *         *         *         *         *         *         *
TATCAGGGAA AAGGAGTAGG GAGTTCATCT GGAGGGTTCT AAGTGGGAGA AGGACTATCA ACACCACTAG GAATCCCAGA

>BamH1
  |
  |                                                         1840
  |      *         *         *         *         *         *         *         *
GGTGGATCCT CCCTCATGGC TCTGGCACAG TGTAATCCAG GGGTGTAGAT GGGGGAACTG TGAATACTTG AACTCTGTTC

                                                            1920
         *         *         *         *         *         *         *         *
CCCCACCCTC CATGCTCCTC ACCTGTCTAG GTCTCCTCAG GGTGGGGGGT GACAGTGCCT TCTCTATTGG CACAGCCTAG

                                                            2000
         *         *         *         *         *         *         *         *
GGTCTTGGGG GTCAGGGGGG AGAAGTTCTT GATTCAGCCA AATGCAGGGA GGGGAGGCAG ATGGAGCCCA TAGGCCACCC

         *         *         *         *         *
CCTATCCTCT GAGTGTTTGG AAATAAACTG TGCAATCCCC TCAAAAAAAA AA
```

FIG. 3C

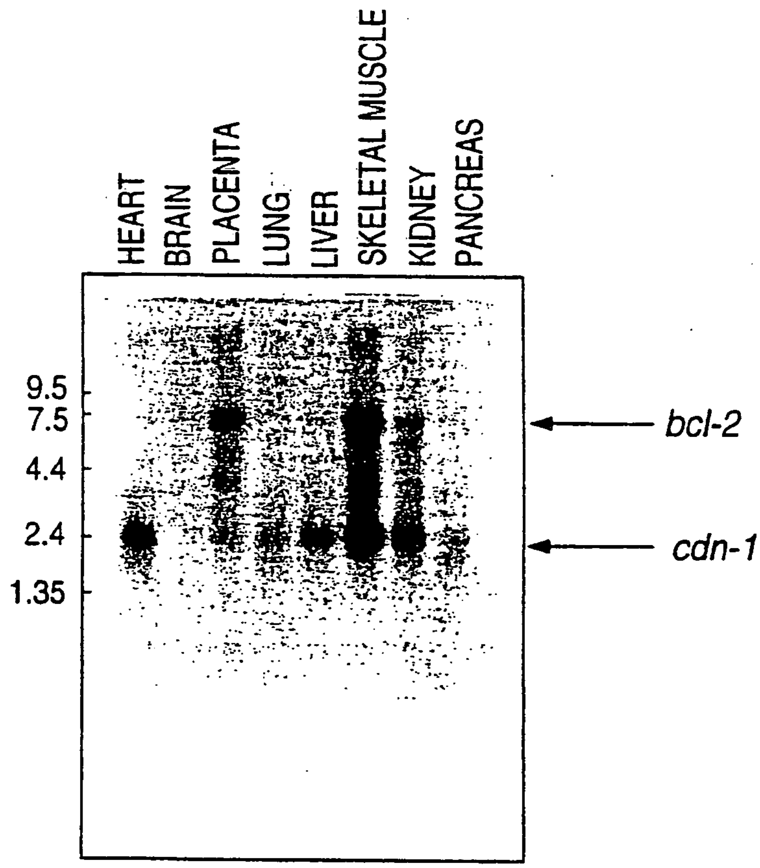

HEART
BRAIN
PLACENTA
LUNG
LIVER
SKELETAL MUSCLE
KIDNEY
PANCREAS
9.5
7.5
4.4
2.4
1.35
bcl-2
cdn-1

FIG. 4

```
>EcoR1                              >BamH1
                                                                                     80
GAATTCCCGG CCGCAATTAA CCCTCACTAA AGGGATCCTC CTGCCTTGGT CTCCCAAAAT GTTGAGATTA TAGGCATGAG

                                                                                    160
CCCACCACGC CTGGCTGGGG TTTTGTTTTT GTTTTTTTAA CATTTGTCAC ATTTCACAAA GGTATTTCAG AATCTCTGAG

                                                                                    240
AAAAGTGCTA TAATGTCTAA TGATACTTTA TATTTGGACA GCACTTTCGT TTGTTTTTTT TGGCGGGGGG GGTGGGAGAA

                                                                                    320
GTCAAGTAAC TTACATATAG TGAAATTTAC CCTTCTTGAG TATGCAGTTC AGTGAGTTTT GATAAATGTG TAATGGTAGT

                                                                                    400
GTAATCACTA CCACAGTCAA GACATGGACA ATTTTCATTA CCCCACGAAG GTCCCTCATG TGTGGTTAGA GTCAGCCCTC

                                                                                    480
CCATCAGCAC AGTCCTGGCA GCCACTGACC TGGTTTCTGT CCCTACTGTT TTGCCTTTTC CAGAATGTCA TTTAAGTGAC

                                                                                    560
ATCATTCATT ATGGAGACTT GTTTTATTTT TTATTTTTTA TTTTTTGAGA AGGAGTCTCG CTCTTGTTGC CCAGGCTAGA

                                            >Sma1
                                                                                    640
GTGCAATGCT GTGATTTCGG CTCACTGCAA CCTCCGCCTC CCGGGTTCAA GTGATTCTCC TGCCTCAGCC TCCCGAGTAG

                                                                                    720
TTCGGGACCA CAGGCGTACA CCACCATGCC CAGCTAATTT TTTTTTTTTT TGAGATGGAG TCTCGCCCTG TCACCCAGGC

                                                       >Sma1
                                                                                    800
TAGAGTCCAG TGGCATGATC TCGCCTCACT GCCAAGCTCC TGCCTCCCGG GTTTCACGCC ATTCCCCTGC CTCAGCCTCC

                                                                 >Bal1
                                                                                    880
CGAGTATGCC CGGCTAATTT TTGTATTTTT AGTAGAGACG GGGTTTCCCC ATGTTGGCCA GGCTAGTCTC AAACTCCTGA
```

FIG. 5A

```
                                                                                       960
CCTCAAGTAA TCCGCCTGCC TTGGCTCCCA AAGTGCTGGG ATTACAGGTG TGAGCCACCG CGCCCAGCCC ATTATGTAGC

                                                              >Spe1
                                                                                      1040
TTTTTGTGCC CCACTTCTCC CACTTAGCAT AATGCTTTTT GAGATTCATC TGTATTACTA GTGCATCTGT AGTTCTTTCC

                                                                                      1120
TTTTTATGGC TTGGGTTGTT TTTTGTTTTT GTTTTTGTTT TTGAGATAGG GTCTCACTCT GTTGCCCAGG GTGGAGTGCA

                                           >EcoR5
                                                                                      1200
CTATCGCAGC TCTCCGCAAC CTCCACCTCC CAGGCTCAAG ATATCCTCCC ACCTCAGCCT CCTGAGTAGC TGGAAATACA

                                                                          >Nco1
                                                                                      1280
AGTGTGTGTG CCACCATGCC GGTTAATTTT TTTTTCTTTT TTTTTTTTTT TTTTCAATTT TTGTTGGAAG CACCATGGAG

                                                 >Bal1
                                             >Sph1
                                                                                      1360
CCGCCTGAGC CTGGCTGAGC CTAAAAGCCC TGTGGTGCAT GCCTGGCCAA TTTTTGTATT TTTTAGTAGA GACGGGATTT

                                                                                      1440
TGCCATGTCG CCCAGGCTGG TCTGGAACTC CTGGTCTCAG GTGATTCTCC TGCTTCGGCC TCCCAAGTAG CTGGGGTTAC

                                                                                      1520
AGGCATGTGC CACCATGCTC AGCCCTCCCG TCAGCACAGT CCTGGCAGCC ACTGGCCTGG TTTCTGTCCC TACTGTTTTG

                                                                                      1600
CCTTTTACTG GTCTCCATGC TCACCTAAAT TTTTTTTTAT TTTTTGTAGA GACAGATTCT CGCAATGTTG CTCAGGCTAG

                                                                                      1680
TCTCGAACTC CCGGCTTCAA GCAATCCTCC CACCTCAGTC CTCCAAAGTT CTGGAATTAC AGGCATGAAT CACTGTGCCA
```

```
                                                >Aha3
                                                  |
                                                >Dral
                                                  |                                              1760
          *          *          *          *      |   *          *          *          *          *
GTGTTGCATT CCAAAGCATG GATACATCAC AGTTTTTAAA ATGTTTACCA ATGTAAATGG CCCGTTTTAA TATGAGAATA

                                                                                              1840
          *          *          *          *          *          *          *          *
ACTAATGTTG AAGAGAGTGC TACAAAGAGG ATCATGGTCG TGATGTGTAG ACACAAAAAT GAGGTTAGAA GACAGTAAGG

                                                                                              1920
          *          *          *          *          *          *          *          *
TGAGGGCCGG GCACAGTGGC TCATGCTTGT AATCCTAGCA CTTTGGGAGG TGCAGGTGGG AGGATTGCTT GAGGCCAGGA

                                                                                              2000
          *          *          *          *          *          *          *          *
GCTGGAGACA AGTCCAGGCA ACACAGCGAG TCCCTGCCTT TATAAAAAAT CAGAAATTAA AAAAGCCTTG GCGGTGGCTC

                                                                                 >Ball
                                                                                   |      2080
          *          *          *          *          *          *          *    |     *
ACGCCTGTAA TCCCAGCACT TTGGGAGGCC GAGACGGGTG GATCACGAAG TCAGGAGTTC AAGACCAGCT TGGCCAAGAT

                                                                                              2160
          *          *          *          *          *          *          *          *
GGTGAAACCC TGTCTCTACT AAAAATAAAA AAAAAAAATT AGCCAGTCGT GGTGGTGGCA CCTGTAATCC CAGCTACTCA

                                                                                              2240
          *          *          *          *          *          *          *          *
GGAGGCTGAG GCAGGAGAAT CGCTTGAACC CAGGAGGCGG AGTTTGCAGT AAGCCAAGGT GCGCCACTGC ACTCCAGCCT

                                                                                              2320
          *          *          *          *          *          *          *          *
GGGCAACAGA GTAAGACTCT GTCTCAAAAA AAAACAAAAA ACAAAAAAAC AAAAAAAAAA CAGGCCGGCG CAGTGGCTCA

              >Scal                                                              >Ball
                |                                                                  |      2400
          *     |    *          *          *          *          *          *      |   *
TGCCTATAAT CCAAGTACTT TGGGAGGCCA AGGCAGGCGG ATCGCAAAGT CAGGAGTTCG AGACCAGCCT GGCCAATATG

                                                                                              2480
          *          *          *          *          *          *          *          *
GTGAAACCCT GTTTCTGCTA AAAATACAAA AAATAGCCAG GTGTGGTGGG AAGCGCCTGT AGTCCCAGCT ACTCAGGAGG

                        >Smal
                          |                                                                   2560
          *          *    |     *          *          *          *          *          *
CTGAGGCAGG AGAATCGCTT GAACCCGGGA GGCAGAAGTT GCAGTGAGCT GAGATTGCGC CACTGCACTC CAGCCTGGGC
```

FIG. 5C

RECTIFIED SHEET (RULE 91)

```
                                                                                >Aha3
                                                                                  |
                                                                                >Dra1
                                                                                  |       2640
      *          *          *          *          *          *          *         |          *
AACAGAGCGA GACTCCATCT CAAAGAAAAA AAAGCCAAAA CATAGTAAGG TGAGGGTGAA ACTTCTCTTT TAAAAAAATG

                                                                                          2720
      *          *          *          *          *          *          *          *
TTTACATAGA AACAAACTAA ATGGACAAAA TGGATATAAA CAAAAATGTT ATCGGTGGTT ATTTTTGGGC AGTAGAATTA

                                                                                          2800
      *          *          *          *          *          *          *          *
TAGGTTTTTA ATTTCTTTTG CTTATTTATA GTTTCAAAAA TTTTCAATTT TAATATAAAT TAATGTGCTC TATTTATAGA

                                                                                          2880
      *          *          *          *          *          *          *          *
GACAATACAT GAAATATACT TAATAAAAAT TCAAATGTTA TAGAACTGAA AAAGATGAAA AGTAAAAACA ACCTATTCCC

                                                                                          2960
      *          *          *          *          *          *          *          *
CAGAGGTAGC CACTGTCCAT AGTTTCTATT TTAGATTCTT TCCTTTATAC AAGATTATTA TAGCTTCTAT TTTTTGGTGT

                                                                                          3040
      *          *          *          *          *          *          *          *
ATGAACTGTA GTCCTAGAGG ATTTTATTAG TTATGAGTTC TATAACTAAG ATCCATCATC TTAGTTGCTA AGAACGTAGA

              >Dra1
                |
              >Aha3
                |                                                                         3120
      *         |*          *          *          *          *          *          *
TACTGAGAAC ATCATTTAAA AAAACATTTT TGGCTGGCAC CTCTATGATC ACTGGAGTCT CGCGGGTCCC TCAGGCTGCA

                                           >Aha2
                                             |                                            3200
      *          *          *          *     |    *          *          *          *
CAGGGACAAG TAAAGGCTAC ATCCAGATGC TGGGAATGCA CTGACGCCCA TTCCTGGAAA CTGGGCTCCC ACTCAGCCCC

                                                              >Sma1      >BamH1
                                                                |           |             3280
      *          *          *          *          *           |  *        |*          *
TGGGAGCAGC AGCCGCCAGC CCCTCGGGAC CTCCATCTCC ACCCTGCTGA GCCACCCGGG TTGGGCCAGG ATCCCGGCAG

      *          *          *          *          *          *          *
GCTGATCCCG TCCTCCACTG AGACCTGAAA A ATG GCT TCG GGG CAA GGC CCA GGT CCT CCC AGG CAG GAG
                                   M   A   S   G   Q   G   P   G   P   P   R   Q   E>
```

```
         3360
           *         *         *          *         *         *
TGC GGA GAG CCT GCC CTG CCC TCT GCT TCT GAG GAG CAG GTA GCC CAG GAC ACA GAG GAG GTT TTC
 C   G   E   P   A   L   P   S   A   S   E   E   Q   V   A   Q   D   T   E   E   V   F>

                  3440
     *         *         *          *         *         *          *
CGC AGC TAC GTT TTT TAC CAC CAT CAG CAG GAA CAG GAG GCT GAA GGG GCG GCT GCC CCT GCC GAC
 R   S   Y   V   F   Y   H   H   Q   Q   E   Q   E   A   E   G   A   A   A   P   A   D>

                                               >NcoI
                                                 |
                                               3520
         *         *          *         *         *         *
CCA GAG ATG GTC ACC TTA CCT CTG CAA CCT AGC AGC ACC ATG GGG CAG GTG GGA CGG CAG CTC GCC
 P   E   M   V   T   L   P   L   Q   P   S   S   T   M   G   Q   V   G   R   Q   L   A>

                                                                                  >PstI
                                                              3600                  |
 *         *          *         *         *          *         *         *          |
ATC ATT GGG GAC GAC ATC AAC CGA CGC TAT GAC TCA GAG TTC CAG ACC ATG TTG CAG CAC CTG CAG
 I   I   G   D   D   I   N   R   R   Y   D   S   E   F   Q   T   M   L   Q   H   L   Q>

                  >ScaI_
                    |
                    |                                                          3680
     *         *    |*         *          *         *         *          *
CCC ACG GCA GAG AAT GCC TAT GAG TAC TTC ACC AAG ATT GCC TCC AGC CTG TTT GAG AGT GGC ATC
 P   T   A   E   N   A   Y   E   Y   F   T   K   I   A   S   S   L   F   E   S   G   I>

         *         *          *         *         *          *
AAT TGG GGC CGT GTG GTG GCT CTT CTG GGC TTC AGC TAC CGT CTG GCC CTA CAC ATC TAC CAG CGT
 N   W   G   R   V   V   A   L   L   G   F   S   Y   R   L   A   L   H   I   Y   Q   R>

         3760
     *         *         *          *         *         *          *
GGC CTG ACT GGC TTC CTG GGC CAG GTG ACC CGC TTT GTG GTG GAC TTC ATG CTG CAT CAC TGC ATT
 G   L   T   G   F   L   G   Q   V   T   R   F   V   V   D   F   M   L   H   H   C   I>

                           3840
         *         *         *          *         *          *
GCC CGG TGG ATT GCA CAG AGG GGT GGC TGG GTG GCA GCC CTG AAC TTG GGC AAT GGT CCC ATC CTG
 A   R   W   I   A   Q   R   G   G   W   V   A   A   L   N   L   G   N   G   P   I   L>

                                                 3920
 *         *          *         *         *         *          *
AAC GTG CTG GTG GTT CTG GGT GTG GTT CTG TTG GGC CAG TTT GTG GTA CGA AGA TTC TTC AAA TCA
 N   V   L   V   V   L   G   V   V   L   L   G   Q   F   V   V   R   R   F   F   K   S>
```

FIG. 5E

RECTIFIED SHEET (RULE 91)

```
                                                    >Afl2
                                                     |   4000
      *       *       *       *       *              |    *       *       *
TGA CTC CCAAGGGTGC CCTTTGGGGT CCCAGTTCAG ACCCCTGCCT GGACTTAAGC GAAGTCTTTG CCTTCTCTGC
 *>

                                    >Hind3                               >Apa1
                                     |                          4080      |
      *         *         *          |    *         *         *           |  *         *
TCCTTGCAGG GGTCCCCCCT CAAGAGTACA GAAGCTTTAG CAAGTGTGCA CTCCAGCTTC GGAGGGCCCC TGTGTGGGGG

           >Pst1            >Nco1            >Apa1        >Apa1     >Aha2
             |                |                |    4160    |         |
      *      |  *         *   |     *         *|  *   *     |     *   |    *
CCAGTCAGGC TGCAGAGGCA CCTCAACATT CCATGGTGCT AGTGGGCCCT CTCTCTGGGC CCAGGGGCTG TGGCGTCTCC

                                                                4240
      *         *         *         *         *         *         *         *
TCCCTCAGCT CTCTGGGACC TCCTTAGCCC TGTCTGCTAG GCGCTGGGGA GACTGATAAC TTGGGGAGGC AAGAGACTGG

                                                                4320
      *         *         *         *         *         *         *         *
GAGCCACTTC TCCCCAGAAA GTGTTTAATG GTTTTAGCTT TTTATAATAC CCTTGTGAGA GCCCATTCCC ACCATTCTAC

         >Aha2
          |                                                     4400
      *   |     *         *         *         *         *         *         *
CTGAGGCCAG GACGTCTGGG GTGTGGGGAT TGGTGTGTCT ATGTTCCCCA GGATTCAGCT ATTCTGGAAG ATCAGCACCC

                                                                4480
      *         *         *         *         *         *         *         *
TAAGAGATGG GACTAGGACC TGAGCCTGGT CCTGGCCGTC CCTAAGCATG TGTCCCAGGA GCAGGACCTA CTAGGAGAGG

                                                                4560
      *         *         *         *         *         *         *         *
GGGGCCAAGG TCCTGCTCAA CTCTACCCCT GCTCCCATTC CTCCCTCCGG CCATACTGCC TTTGCAGTTG GACTCTCAGG

                                                                4640
      *         *         *         *         *         *         *         *
GATTCTGGGC TTGGGGTGTG GGGTGGGGTG GAGTCGAGAC CAGAGCTGTC TGAACTCATG TGTCAGAAGC CCTCCAAGCC

                                                                4720
      *         *         *         *         *         *         *         *
TGCCTCCCAG GGTCCTCTCA GTTCTCTCCC TTCCTCTCTC CTTATAGACA CTTGCTCCCA ACCCATTCAC TACAGGTGAA
```

FIG. 5F

```
                                    >Stu1
                                     |              4800
     *         *         *         * |       *         *         *         *
GGCTCCTCAC CCCCATCCCT GGGCCTTGGG TGAGTAACCT GCTAAGGCCT CCTTGCCCAG ACTACAGGGC TTAGGACTTG

                                                    4880
     *         *         *         *         *         *         *         *
GTTTGTTATT TCAGGGAAAA GGAGTAGGGA GTTCATCTGG AGGGTTCTAA GTGGGAGAAG GACTATCAAC ACCACTAGGA

          >BamH1
           |                                        4960
     *     |   *         *         *         *         *         *         *
ATCCCAGAGG TGGGATCCTC CCTCATGGCT CTGGCACAGT GTAATCCAGG GGTGTAGATG GGGGAACTGT GAATACTTGA

                                                    5040
     *         *         *         *         *         *         *         *
ACTCTGTTCC CCCACCCTCC ATGCTCCTCA CCTGTCTAGG TCTCCTCAGG GTGGGGGGTG AGAGTGCCTT CTCTATTGGG

                                                    5120
     *         *         *         *         *         *         *         *
CACAGCCTAG GGTCTTGGGG GTCGGGGGGA GAAGTTCTTG ATTCAGCCAA ATGCAGGGAG GGGAGGCAGA TGGAGCCCAT

                                                    5200
     *         *         *         *         *         *         *         *
AGGCCACCTC CTATCCTCTG AGTGTTTGGA AATAAACTGT GCAATCCCCT CAAAAAAATA AAAATAAAAA AAATAAAAAT

                                                    5280
     *         *         *         *         *         *         *         *
AAAAAAACAT TTTTTTCAAG CAGGGAGTGG TGGCTCCCGC CTGTAATCCC AGCACTTTGG GAGGCCAAGG CGTGCAGATT

                                                    5360
     *         *         *         *         *         *         *         *
GCTTCAGTTC AGGAGTTCAA GACCAGCCTG GGAAACATGG TGAAACCCCA TCTCTACTAA AAATAAAAAA TTAGCCAGGC

                                                    5440
     *         *         *         *         *         *         *         *
ATAGTGTCGC GCACCTGTAC TCCCAGCTAT TTGGGAGGCT GAGGTAGGAG AATTGCTTGA ACCCAGGAGG TGGAGGTTGC

                                                    5520
     *         *         *         *         *         *         *         *
AGTGAGCTGA GATCAGGCCA CTGCACTCCA ACGTAGGTGA CAGAGATAGC CTCCTTCTAA AAAAACAACC TTTTTTCCAG

                                      >Xba1
                                       |            5600
     *         *         *         *   |     *         *         *         *
CCAAAACAAC TGAACTTCCT CCCCACTGAC CACCTCAATT ATTTCTAGAT GCCTTGTTGC TGTCCAGACT GCGGTGATTC

                                                    5680
     *         *         *         *         *         *         *         *
CCTGGGCTGA TCTGAGCCCG TGGCCTGAGT CATTTGCAGT TCCTCTAGCA GGTGGTCCCC CATGTCATGG CCCCTGTGAA
```

FIG. 5G

```
                                                                    >Hind3
                                                       5760              |
         *         *         *         *         *         *         *   *
ACCAGTTCCT TACCATCTCT GTTCATCGCT GCTCCCTAAG TTAGGCCCTG CATGTCTTGA GGGTAGGTTA GATTCAGAAA

                                                       5840
         *         *         *         *         *         *         *         *
AGCTTTGGTC GCATCACTGC TTTCATAAAC TCAAATGAGA GGGAGGGAGG GAAGGCAGGA AGAAGGGAGG GAGTCCTTTC

                                                       5920
         *         *         *         *         *         *         *         *
TCTCCCACAG TGTGCATTAC CTCATGTAAC ACTTCTTGCT AATGTGGTAG AATGTGTTTG ACTTTGAATG AGACTTGGGT

                                                       6000
         *         *         *         *         *         *         *         *
TTATTTTTAT TTATTTATTT ATTTATTTAT TTATTTATTT TGAGATGGAG TTTCACTCTT GTTGCCCAGG CTGGAGTGTA

                                                       6080
         *         *         *         *         *         *         *         *
GTGGCACGAT CTCTACTCAT TGCACCCTCC GCCTTCCAGG TTCAAACGAT TCTCCTGCCT CAGCCTCCCA AGTAGCTGGG

           >Sph1
             |
             |                                         6160
         *   |     *         *         *         *         *         *         *
ATTACAGGGG CATGCCACCA TGCCCAGCTA ATTTTTGTAT TTTTAGTAGG GACGGGGTTT CACCATGTTG ACCAGGCTGG

                                                       6240
         *         *         *         *         *         *         *         *
TCTGGAACTC CTGATCTCAG GTGATCCACC TGCCTCGGCC TCCCAAAGTG TTGGGATTAC AGGCGTGAGC CACCGTGCCT

                                                       6320
         *         *         *         *         *         *         *         *
GGCCTGAGAC TTAAATCCAT CTCTTTTTTC TTCTTCTTTT TGAGACAGAG CCTCATTCTG TTCCCCATGC TGGAGTTCAG

                            >Bal1
                             |
                             |                         6400
         *         *         *         *         *         *         *         *
TGGCGTGATT TTGGCTCACT GCAACCTTGG CCATCTGGGT TTGAGCAATT CTCGTGCCTC AGCCTCCTGA GTAGCTGGCA

                                                       6480
         *         *         *         *         *         *         *         *
CTATAGTCAC ATGCCACCAC GCCCGGCTAA CTTTTTTGTA TTTTTAGTAG AGACAGGGTT TCACTATGTT AGCCAGGCTG

  >EcoR1
     |
     |   *
GTCTCGAATT C
```

FIG. 5-

```
cdn1                                                 masgqgpgpprqecgepalpsaseeqvaqdteevfrsyvfyrhqqeqeaegvaapadpemvt
cdn2                                                 masgqgpgpprqecgepalpsaseeqvaqdteevfrsyvfyHhqqeqeaegAaapadpemvt
bcl2                                  mahagrtgyDNREIVMKYIHYKLSQRGYEWdagdvgaappgaapapgifssqpghtphtaasrdpvartsplqtpaapgaa
bax                                                                             mdgsgeqprgggptsseqimktgalllqgfiqdragrmggeap
bcl-x                                             msqSNRELVVDFLSYKLSQKGYSWsqfsdveenrteapegtesemetpsaingnpswhladspavngatghsssl
mcl-1                              ...(+123 aa)eldgyepeplgkrpavlpllelvgesGnntstdgslpstpppaeeeedelyrqsleiisrylreqatgakdtk
A1                                                                                                    maeselmhihslaehylqyvlq
bhrf                                                                                         maystreillalcirdsrvhgngtlhpvlelaar
LMW5-HL                                                                                                   megeeliyhniineilvgy
ced9       mtrctadnsltnpayrrrtmatgemkeflgikgteptdfginsdaqdlpspsrqastrrmsigesidgkindweeprlDIEGFVVDYFTHRIRQNGMEWfgapg

cdn1       lplqpsstmgQVGRQLAIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIATSLFESGI-NWGRVVALLGFGYRLALHVYQHGLTGFLGQVTRFVVDFMLHH
cdn2       lplqpsstmgQVGRQLAIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIASSLFESGI-NWGRVVALLGFSYRLALHIYQRGLTGFLGQVTRFVVDFMLHH
bcl2       agpalspvppVVHLTLRQAGDDFSRRYRRDFAEMSRQLHLtpftargRFATVVEELFRDGV-NWGRIVAFFEFGGVMCVESVNREMSPLVDNIALWMTEY-LNR
bax        elaldpvpqdastkklseclkrigdeldsnmelqrmiaavdtdsprevFFRVAADMFSDGNFNWGRVVALFYFASKLVLKALCTKVPELIRTIMGWTLDF-LRE
bcl-x      darevipma-AVKQALREAGDEFELRYRRAFSDLTSQLHITPGTAYQSFEQVVNELFRDGV-NWGRIVAFFSFGGALCVESVDKEMQVLVSRIAAWMATY-LND
mcl-1      pmgrsgatsrkalETLRRVGDGVQRNHETVFQGMLRKLDIKNEDDVKSLSRVMIHVFSDGVTNWGRIVTLISFGAFVAKHLKTINQESCIEPLAESITD-VLVR
A1         vpafesapsqacrvlqrvafsvqkeveknlksylddfhvesidtariiFNQVMEKEFEDGIINWGRIVTIFAFGGVLLKKLpqeqialdvcaykqvssfvaefi
bhrf       etplrlspedtvvlryhvlleeiiernsetftetwnrfithtehvdldfnsvfleifhD-LINWGRICGFIVFSARMAKYCKDANn-HLESTVITTAYNF-SEG
LMW5-HL    ikyymndihelspyqqqikkiltyydeclnkqvtitfsltnaqeiktQFTGVVTELFKrgdpslgralawmawcmhacrtlccnqstpyyvvdlsvrgmleaM-
ced9       lpcgvqpehemmrvmgtifekkhaenfetfceqLlavprisfslyqdvvrtvgnaqtdqcpMSYGRLIGLISFGGFVAAKMmesvelqgqvrnlfvytslfIKT

cdn1       CIAR--WIA-QR-GGWVAALNLGngpilnvlvvlgvvllgqfvvrrffks
cdn2       CIAR--WIA-QR-GGWVAALNLGngpilnvlvvlgvvllgqfvvrrffks
bcl2       HLHT--WI--QDNGGWDAFVELYgpsmrplfdfswlslktllslalvgacitlgaylghk
bax        RLLG--WI--QDQGGWDGLLSYFgtptwqtvtifvagvltasltiwkkmg
bcl-x      HLEP--WI--QENGGWDTFVELYgnnaaaesrkgqerfnrwfltgmtvagvvllgslfsrk
mcl-1      TKRD--WLVKQ--RGWDGFVEFFhvedleggirnvllafagvagvgaglaylir
A1         MNNTGEWI-RQ-NGGWEdgfikkfepksgwltflqmtgqiwemlfllk
bhrf       -LDG--WIHQQ--GGWStliednipgsrrfswtlflagltlsllvicsylfisrgrh
LMW5-HL    KHNLLPWMISH--GGQEEFLAFslhsqiysvifnikyflskfcnhhflrscvqllrkcnli
ced9       -RIRNNWKE-H-NRSWDDFMTLgkqmkedyeraeaekvgrrkqnrrwsmigagvtagaigivgvvvcgrmmfslk
```

SEQUENCE IDENTITY:

cdn1/cdn2 = 97%

FIG. 6

```
>EcoR1
|
|        *         *         *         *         *         *         *        80
|                                                                            *
GAATTCTGGT AATTAGTTAA ACAACCTTGA ACAAGTTGTT TCACTTCTCT GAGTCTCAGT TTCTCACTCA AAAATGGTGA

         *         *         *         *         *         *         *       160
                                                                             *
ATAATTTGTA AGACTTCGCT AATAATCTAC GACTCTACAA GAGGCAATAG GGTACTGTGG ACAGAGAGCA GGCTTTGGAA

                                                  >Hind3
                                                >Ball|
                                                    ||
         *         *         *         *         *  ||     *         *       240
                                                                             *
ACACACAAGA CTGGGTTTAG ATTCCTGCAC TCCACCCAGT GTGTGACTTG GCCAAGCTTC TTCACTTCTC TAAACCCCCA

         *         *         *         *         *         *         *       320
                                                                             *
TCTGTGTATC TGTACAGGAA TGAATGAATG AGTATGTGCA GCCAAGCTAT GCAAACTCCA GGTTAAAATA TTGCCTTGGG

                                                            >Afl2
                                                             |
         *         *         *         *         *         * *         *       400
                                                                             *
TTTTTTAGTA AATTGTTCAA GCCCATGACA TTCTAGCAGA AAAAGCCTAG TGTCTCTTTC TTAAGGTGAT TGTGTCCATG

         *         *         *         *         *         *         *       480
                                                                             *
TGTTTTCCAG GAACTCTATG GGTTTCTCAA CCCAAATTCA CCCTGCCCTT GACCAAATGG CTCACCAGCT TCACGGATGC

                    >Pst1                >Pst1
                     |                     |
         *         * |       *         *   |     *         *         *       560
                                                                             *
TGCTCTGATG ACACACCCTG CAGTCAGCAT CTGCCCCTGC AGCTAGAATG GATTTCTGAG TGGGCATTAG CTGGGGGATA

                    >Bgl2
                     |
         *         * |       *         *         *         *         *       640
                                                                             *
CCACATGGGC ACCAATGTCA CAGATCTTCT GTCACAGTCC ACCCCGAACC ATTGCTTCTC AAATCATAAT CCCTTAGCAG

                                                                         >Spe1
                                                                           |
         *         *         *         *         *         *         *     | 720
                                                                             *
GACAGCTAGG TGCAGCACGC ATGACACAAA CACCAGCCCT TGCCTACAAT CTCAGCCACT ATCTTGAGTC TGAGCAACTA

         *         *         *         *         *         *         *       800
                                                                             *
GTCTAGTGGC AGCCGCGCCC TTCCTTTTCA AGAGAGTTCT GGGATCAGAT CCTTTCACAA ACAGATCCCT CCCCACCCTG

         *         *         *         *         *         *         *       880
                                                                             *
[illegible] ACGTCTGCAC ACTGAAAAGT AAGACAGCAT TTGCTAAGCC ATATTTCAAA AAGTTTGCTT ATACCTTCAT
```

```
                >Afl2                                                                 >Stul
                 |                                                                     |  960
          *     *|    *         *         *         *         *         *            |  *
CTCAGGACAA CAAGTGCCTG CTTAAGAGCC TTATGTTTGT GTAACTGGTA TTTTTTTTTC CCCTGACCTT CCAAGGCCTA

                                                                                        1040
          *         *         *         *         *         *         *         *
GTCTACTTTC TCCCTCCCTA GCTGAACAAA AGTGAAGTTG AAATAATTTG AACTACCCCT TTTAGTGGGC AGCCCATTTG

                                                                                        1120
          *         *         *         *         *         *         *         *
ATTTTTACCT TAGCCAGAGC CTTAATTTGT CCATGTGAGC ATAGCAGTAC CTTGCAGCAC CTGAGGCACA ATACATTGTT

>Dra1
 |
>Aha3                                                                              >Nco1
 |                                                                                   |
 |                                                                                  1200
 |        *         *         *         *         *         *         *           | *
TAAAGAGTGA CAGTGCGTCC CATTCCAATA AGAACCACAC TCAGAGCAAA GGTTCCCTCT CCTGTGTGGA GAGTGACCCA

                                                                                        1280
          *         *         *         *         *         *         *         *
TGGTAGAAAA TTTGCAGACT TCGTTACCTC TTCATCAGTT GAAAAATCTA TTTATTCATT TATGCATTTA ATTTTCCCTA

                                                                          >Pst1
                                                                           |            1360
          *         *         *         *         *         *         *         *
TCTAAGCCAG GGATAGTCAA ACATTTTCTG TAAAGGGCCA AGTAGCATGA TAAATATGTT AGGCTCTGCA GGCCACTTAC

                                                                 >Aha3
                                                                  |
                                                                 >Dra1
                                                                  |                     1440
          *         *         *         *         *         *     |   *         *         *
AGTTTTGTCA TGTATTCTTT TTTTGCTCCC TGTTTGTATT ATTTTGTTTA CAATGCTTTA AAAATGTAAA AAAACAGATG

                                                                                   >Aha2
                                                                                     |
                                                                                    1520
          *         *         *         *         *         *         *           | *
ATCACTGGAG TCTCACGGGT CCCTCGGGCC ACACAGGGAC AAGCAAAGGC TACATCCAGA TACCAGAAAT GCACTGACGC

                                                                                        1600
          *         *         *         *         *         *         *         *
CCGTTCCTGG AAGCTGGGCT CCCACTCAGC CCCTGGGAGC AGCAGCCTCC AGCCCCTTGG GACCTTCAAC TCCACCCTGC

          *         *         *         *         *         *         *
TGACCCACGC GGGTTGAGCC AGCATCCCTG GAGGCTGACA CTGTCCTCCA CTGAGACCTG AAAA ATG GCA TCG GGG
                                                                        M   A   S   G>
```

FIG. 7B

```
1680
  *           *           *           *           *           *           *
CAA GGC CCA GGG CCT CCC AGG CAG GAG TGC GGA AAG CCT GCC CTG CCC TCT GCT TCT GAG GAG CAG
 Q   G   P   G   P   P   R   Q   E   C   G   K   P   A   L   P   S   A   S   E   E   Q>

                  1760
      *           *           *           *           *           *
GTA GCC CAG GAC ATG GAG GGG TTT TCC GCA GCT ACG TTT TTT ACC ACC ATC AGC AGG AAC AGG AGG
 V   A   Q   D   M   E   G   F   S   A   A   T   F   F   T   T   I   S   R   N   R   R>

       >Not1                                                                  >Nco1
         |                                                                       |
         |                             1840                                      |
 *       | *           *           *           *           *           *       * |
CTG AAG GGG CGG CCG CCC CTG CCG ACC CAG AGA TGG TCA CCT TGC CCC TCC AAC CTA GCA GCA CCA
 L   K   G   R   P   P   L   P   T   Q   R   W   S   P   C   P   S   N   L   A   A   P>

                                                       1920
    *           *           *           *           *           *
TGG GGC AGG TGG GAC GGC AGC TCG CCA TCA CCA GGA CGA CAT CAA CCG GCA CTA TGA CTTCGGAGT
 W   G   R   W   D   G   S   S   P   S   P   G   R   H   Q   P   A   L   *>

            >Pst1     >Pst1                          >Sca1_
              |         |                              |
              |         |                              |    2000
         *    |  *      |    *          *          *   |      *          *          *
TCCAGACCAT GCTGCAGCAC CTGCAGCCCA CGGCAGAGAA CGCCTACGAG TACTTCACCA AGATCGCCTC CAGCCTGTTT

                                                            2080
         *          *          *          *          *          *          *          *
GAGAGTGGCA TCAACCGGGG CCGTGTGGTG GCTCTCCTGG GCTTCGGCTA CCGTCTGGTC CTACATGTCT ACCAGCACGG

                                                            2160
         *          *          *          *          *          *          *          *
CTTGACTGGC TTCCTGGGCC TGGTGACCCG CTTCGTGGTC TTCATGCTGC AACAAGGCAT CGCCCGGTGG ATCTCGCAGA

                                                            2240
         *          *          *          *          *          *          *          *
GGGGCGGCTG GGTGGCAGCC CTGGACTTGG GCAATAGTCC CATCCTGAAC GTGCTGGTGG TTGTGGGTGT GGTTCTGCTG

                                                                 >Pvu2
                                                                   |
                                                            2320   |
         *          *          *          *          *          *  |       *          *
GGCCAGTTTG TGGTAAGAAG ATTCTTCAAA TCATGACTCC CAGGGGTGTC CTTTGGGGTC CCAGCTGTGA CCCCTGCCTG

>Af12
 |
 |                                                          2400
 |       *          *          *          *          *          *          *          *
GACTTAAGCC AAGTCTTTGC CTTCCCCACT CCCTTGCAGG GGTCACCCTT CAAAAGTACA GAAGCTCTAG CAAGTGTGCA
```

```
        >Apa1                                                       >Apa1
         |                                           2480            |
         *  |      *         *         *         *         *         *  |  *
CCCCCGCTGC GGAGGGCCCC TGCGTGGGGG CCAGTCAGGC TGCGGAGGCA CCTCAACATT GCACGGTGCT AGTGGGCCCT

   >Apa1                                                        >Pst1
    |                                            2560             |
         *  |      *         *         *         *         *         *  |  *
CTCTCTGGGC CCAGGGGCTG TGCCCTCCTC CCTTGGCTCT CTGGGACCTC CTTAGTCTTG TCTGCTAGGC GCTGCAGAGG

                                                 2640
         *         *         *         *         *         *         *         *
CTGATAACTT GGGGAAGCAA GAGACTGGGA GCCACTCCTC CCCAGTAAGT GTTTAACGGT TTTAGCTTTT TATAATACCC

                                                 2720
         *         *         *         *         *         *         *         *
TTGGGAGAGC CCATTCCCAC CATTCTACCC AAGGCCGGGA TGTCTGGGGT GTGGGGGTTG GTGGGTCGTA ACCTACGTGC

                                                 2800
         *         *         *         *         *         *         *         *
CCCAGGATTC AGCTATTCTG GAAGATCAGA GCCTAAGAGC TAGGACTTGA TCCTGGTCCT GGCCGTCCCT AAGCATCATG

                                                 2880
         *         *         *         *         *         *         *         *
TGTCCCAGGA GCAGGACTGA CTGGGAGAGG GGACCAAGGT CCTACCCAGC TCTCCCCGTG CCCCCATTCC TCCTCCGGCC

                                                 2960
         *         *         *         *         *         *         *         *
ATACTGCCTT TGCAGTTGGA CTCTCAGGGA TTCTGGGCTT GGGGTGTGGG GCGGCGTGGA GTAACAGGCC AGAGCTGTCT

                                                 3040
         *         *         *         *         *         *         *         *
GAACTTATGT GTCAGAAGCC TCCAAGCCTG CCTCCCAAGG TCCTCTCAGC TCTCTCCCTT CCTCTCTCCT TATAGATACT

                                                 3120
         *         *         *         *         *         *         *         *
TGCTCCCAAC CCATTCACTA CAGGTGAAGG CCCTCACCCA TCCCTGGGGG CCTTGGGTGA GTGATGCGCT AAGGCCCCTC

                                                 3200
         *         *         *         *         *         *         *         *
CCCGCCCAGA CTACAGGGCT TGGTTTAGGG CTTGGTTTGT TATTTCAGGG ATAAGGAGTA GGGAGTTCAT CTGGAAGGTT

                                  >BamH1
                                     |           3280
         *         *         *         *     |   *         *         *         *
CTAAGTGGGA GAAGGACTAT CAACACCACA GGAATCCCAG AGGTGGGATC CTCCCTCATG GCTCTGGCAC AGTGTAATCC

                                                 3360
         *         *         *         *         *         *         *         *
AGGGGTGGAG ATAGGGAACT GTGAATACCT GAACTCTGTC CCCCGACCCT CCATGCTCCT CACCTTTCTG GGTCTCTCCT
```

FIG. 7D

SUBSTITUTE SHEET (RULE 26)

```
                                                              3440
          *          *          *          *          *          *          *          *
CAGTGTGGGG GTGAGAGTAC CTTCTCTATC GGGCACAGCC TAGGGTGTTG GGGGTGAAGG GGGAGAAGTT CTTGATTCAG

                                                              3520
          *          *          *          *          *          *          *          *
CCAAATGCAG GGAGGGGAGG CAGAAGGAGC CCACAGGCCA CTCCCTATCC TCTGAGTGTT TGGAAATAAA CTGTGCAATC

                                                              3600
          *          *          *          *          *          *          *          *
CCATCAAAAA AAAAAAGGAG AAAAAAATGT AAAAAACATT CTTAGCTGTA AGCTACTTAT AGGGGGATAA AGACAGGACT

                                                              3680
          *          *          *          *          *          *          *          *
GTTAATGGAC ACAAACATAC AGTTAGAGAG AAGAAATAAG TTCTGTCCAG GCACGGTGGC TCACACCTCT AACTCCAGCA

                                                                     >Bgl2
                                                              3760    |
          *          *          *          *          *          *    |     *          *
CTTTGGGAGA CCAAAGTGGG AAGATCATTT GAGTCCAGGA GTTCGAGACC AGCCTGGACA ACATAGCAAG ATCTTATCTC

       >Dra1
        |
       >Aha3                          >Pst1
        |                              |                      3840
          *          *          *          *          *          *          *          *
TACAGAAAAT TTAAAAAAAA GAAAAAAACT AGCCGCACAG GTCTGCAGTC CTAGCTACTC GGGAGGCTAA GGTGGGAGAA

                                                              3920
          *          *          *          *          *          *          *          *
TCCTTGAACC CAGGGATTTA GTTTGAGGTT GCAGTGAGCT ATGATTGCAC CACTGCACTC CAGACTGGGT GACTGAGTGA

                                                              4000
          *          *          *          *          *          *          *          *
GACCCTGTCT CAAATATAAA GAAGGAACAA GTTCTAGTTT TCAATAGCGC AATAGGGTGA GTGCAGTTAG CAACAACATA

                                                              4080
          *          *          *          *          *          *          *          *
TTGTGTATTT CAAAATAGCT ACAAGAGAGG ATATGAAGTG TTCCCCCAAA CAAGGAATGA TAACGTTCGA GGTGACAGAT

                                                              4160
          *          *          *          *          *          *          *          *
ACCTTAAATA CCCTGATTTG ATCATTACAC ATTCAATGTA TGTATCAAAA TATTACATGT ACCCCACAAA TTTGTGTAAA

                  >Dra1
                   |
                  >Aha3
                   |                                          4240
          *        |  *          *          *          *          *          *          *
TATTATGTAT CCACTTTTTA AAGTTGGCAG AGCCCAAAAG CACTACTATG GCTTCCAGTG GTCACTGTGA GCACTGCCAG
```

```
                                                          4320
     *         *         *         *         *         *         *         *
CTCAGCAAAT GTATCACCCA AAATCTGGGC AATGTGGGAA ATTGGCTTCA TGGCAGCTAT GGCTTTGCCA CTGATAGGAA

                                                          >Bal1
                                                           |
                                                          4400
     *         *         *         *         *         |*        *         *
TGATTTCCAG AGATACTTAA TCCTCAATTC GGGACTCTTT GCTTCAGGAG TTTGGCTGGC CAGGAACATG AGTGACAGTG

              >Pvu2                                                  >Xba1
               |                                                      |
                                                          4480        |
     *         |*        *         *         *         *         |*        *
ACCTCTTGGC ACTTCAGCTG GGGGTGTAGC CAAGCAGACA AATGGAATCT TGTGCTGAAC CCAAACCTTC TAGAAACAGA

                                                    >Pvu2
                                                     |
                                                     |    4560
     *         *         *         *         *       |   *         *         *
GCCTGTGAGC ATCACAAGAT ATGCCCTGAT GGAAGCTGAA GTTTAATTCA GCTGAGCGCT TGCCCCTTTC CAACCTGGTT

                                          >Bal1
                                           |
                                           |              4640
     *         *         *         *       |   *         *         *         *
TCTTTTTGTT CCTTGAGTCC AGTCAGAATG CCATTCCCTG GCCAGCAGCC AGCCTTTAGT GACTGTCTCT GTTCTGCAAA

                        >Pst1
                         |
                         |                                4720
     *         *         |*        *         *         *         *         *
GCTCTGTATA TAGTTACTGA GTTTCTGCAG GGGGTGATCT TTGCTCTTGT CCTAAGAAAT AACTACAGTG TTTTAAGAAA

                                                          4800
     *.        *         *         *         *         *         *         *
TATTTGAGGC CGGGTGCAGT GGTTCACACC TGTAATCCAG CACTTTGGGA GGCCAAGGCA GGTGGATCAT GAGGTCAAGA

              >Bal1
               |
               |                                          4880
     *         | *       *         *         *         *         *         *
GTTTGAGACC ATCATGGCCA ACATGGTGAA ACCCCATCTC TACTAAAAAT ACAAAAATTA GCTGGGTGTG GTGGCGGGCA

                                                                            >EcoR5
                                                                              |
                                                          4960                |
     *         *         *         *         *         *         *          | *
CCTGTAGTCC CAGCTACTCG GGAGGCTGAG GCAGGAGAAT CGCTTGAGCC TGGGAGGCGG AGGTTGCACT GAGCCGATAT
```

FIG. 7F

```
                                                                  5040
          *          *          *          *          *          *          *          *
CACGCCACTG CACTCCAGCC TGGCGACAGA GCGAGACTCC ATCTCAAAAA AAAGAAAAAA TAAATAGTTG AAATAAAGAC

                                                                                >Apa1
                                                                                  |
                                                                  5120            |
          *          *          *          *          *          *          *     |    *
TGCACATAAA GACAAAAAAA AAGTTTATAA AGTTAAAAAA TAAAATAAAA AACAGGCTCC AGGCTGGATT GGGCCCAGAG

                                                                  5200
          *          *          *          *          *          *          *          *
GCTGTAGGAC ACAGACCCCC AGCCAATGAC TTCATAAATC CGGATGTTAA TCAGCCTCAC CTGGGAATTT GGGGAGGGGA

    >Aha3
     |
    >Dra1                                                               >Afl2
     |                                                            5280    |
     |    *          *          *          *          *          *        |  *          *
CTCATTTTAA AACAGTTTCC TGGATTCTAA CCCAACCCAG AAAATCAGAC TCTTTGAGCT AAATTCTTAA GCTCCCTGGT

                                                                  5360
          *          *          *          *          *          *          *          *
GATGATGATG GAACCAGTTT ATGGCTGACC CCAGAGTACC GTCTGAAAGA CGTGCCACAT CCCTCTCTCT CCAGCCTCCC

                  >EcoR1
                    |
          *         *|
CTTCTCCTCC ATTCCCCAGG GAGAATTC
```

FIG. 7G

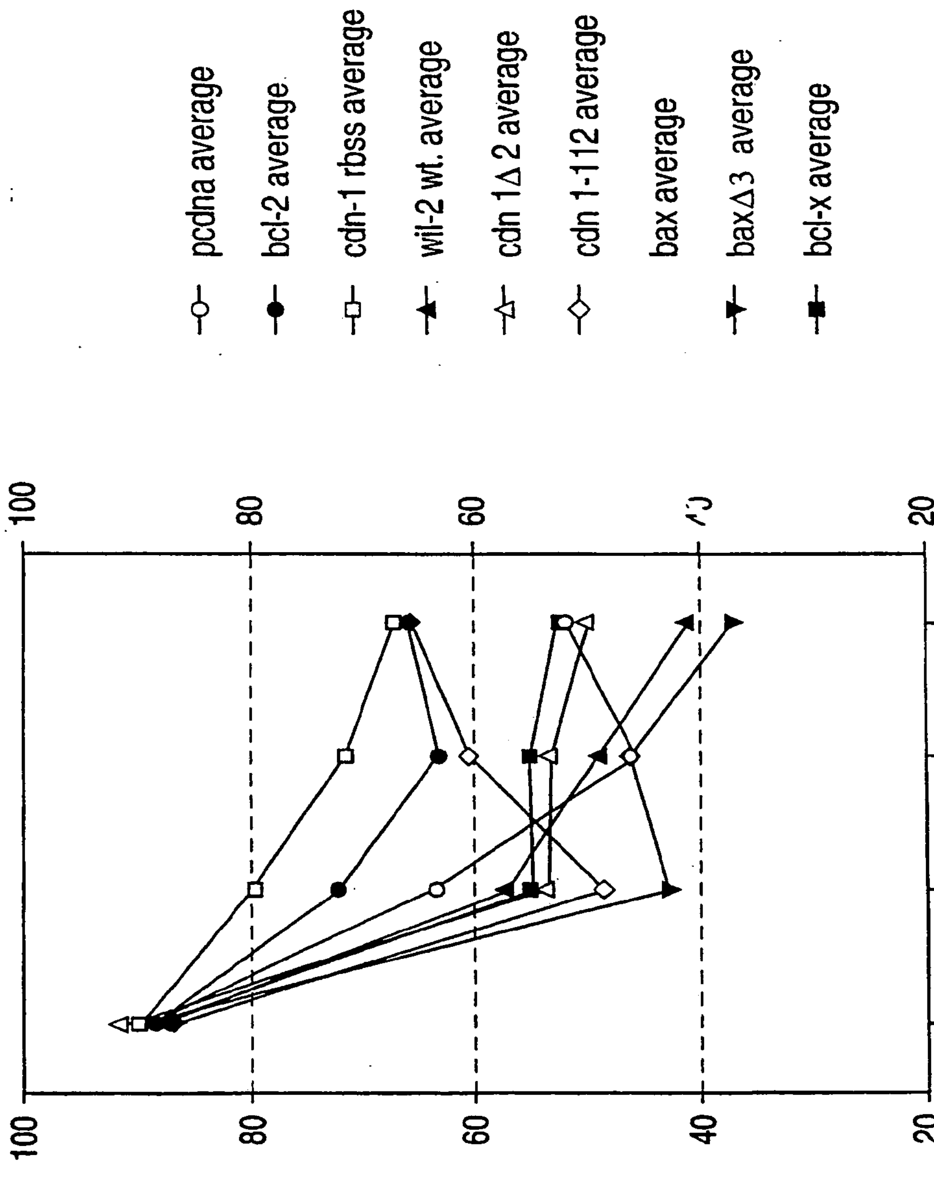

pcdna average
bcl-2 average
cdn-1 rbss average
wil-2 wt. average
cdn 1Δ 2 average
cdn 1-112 average
bax average
baxΔ3 average
bcl-x average
100
80
60
40
20
0 HOURS
24 HOURS
48 HOURS
72 HOURS
COLUMN 1

FIG. 8

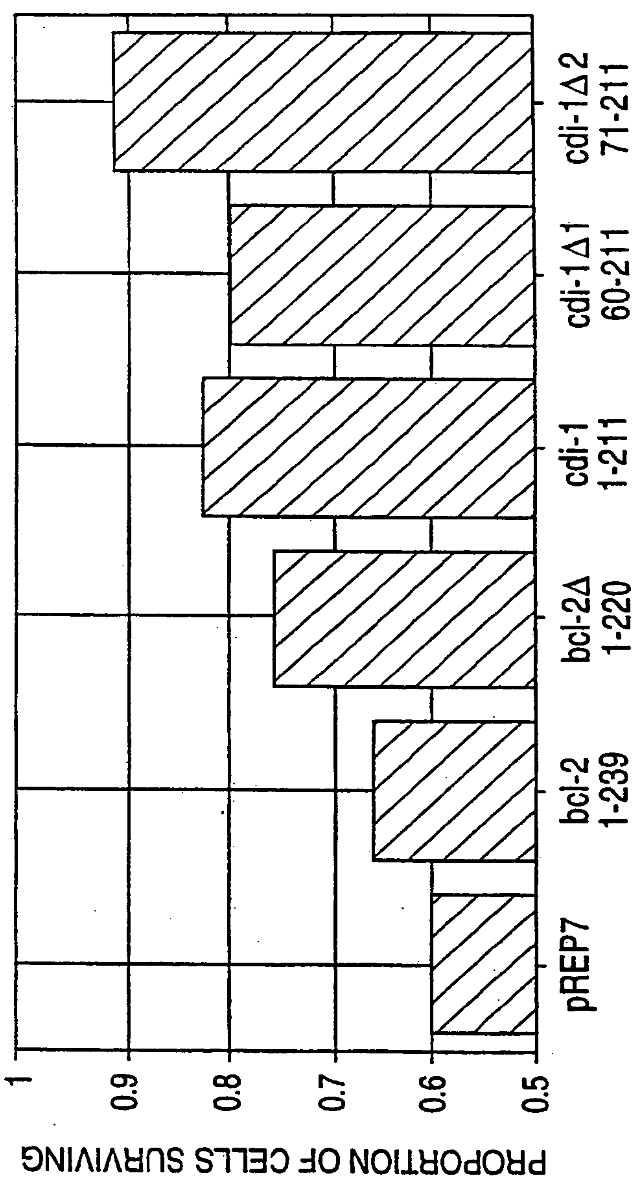

PROPORTION OF CELLS SURVIVING
1
0.9
0.8
0.7
0.6
0.5
pREP7
bcl-2
1-239
bcl-2Δ
1-220
cdi-1
1-211
cdi-1Δ1
60-211
cdi-1Δ2
71-211

FIG. 9

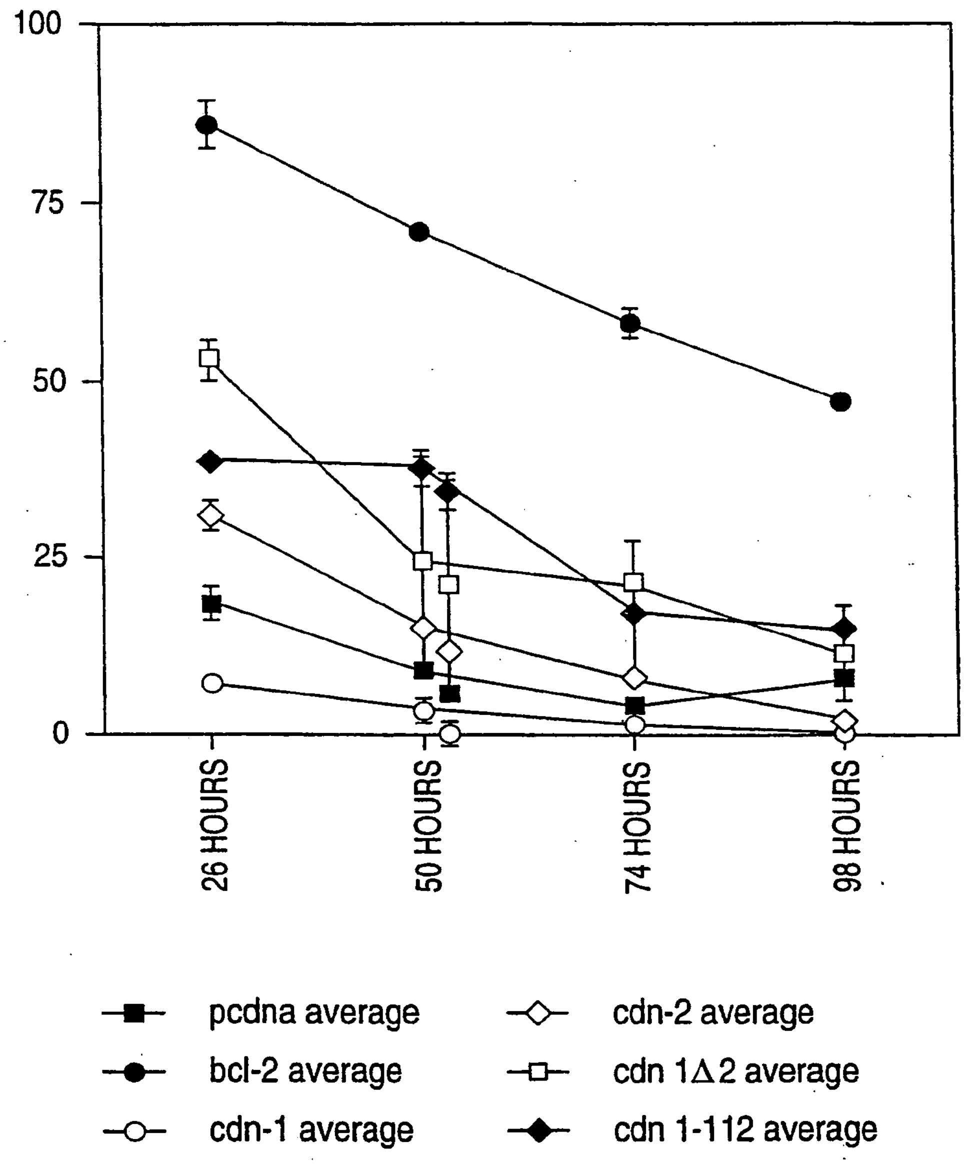

100
75
50
25
0
26 HOURS
50 HOURS
74 HOURS
98 HOURS
pcdna average
bcl-2 average
cdn-1 average
cdn-2 average
cdn 1Δ2 average
cdn 1-112 average

FIG. 10

Δ1

MASGQGPGPPRQECGEPALPSASEEQVAQDTEEVFRSYVFYRHQQEQEAEGVAAPADPEMVT

Δ2 Δ3

LPLQPSSTMGQVGRQLAIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIATSLFESGNWGR

VVALLGFGYRLALHVYQHGLTGFLGQVTRFVVDFMLHHCIARWIAQRGGWVAALNLGNGPILN

VLVVLGVVLLGQFVVRRFFKS

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13930

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.21, 130.1, 141.1; 435/6, 7.1, 7.2, 7.21, 7.24, 7.7, 7.8, 69.1, 70.1, 240.21, 320.1; 514/2, 44; 530/387.1; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CELL, Volume 74, issued 27 August 1993, L.H. Boise et al., "bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death", pages 597-608, see entire document.	1-61
Y	CELL, Volume 74, issued 27 August 1993, Z.N. Oltvai et al., "Bcl-2 heterodimers in vivo with a conserved homolog, Bax, that accelerates programed cell death", pages 609-619, see entire document.	1-61
Y	SCIENCE, Volume 261, issued 09 July 1993, N. Zhu et al., "Systemic gene expression after intravenous DNA delivery into adult mice", pages 209-211, see entire document.	42-58

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search 07 MARCH 1995	Date of mailing of the international search report 20 MAR 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Deborah Freese /for BRIAN R. STANTON Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CELL, Volume 74, issed 10 September 1993, G.T. Williams et al., "Molecular regulation of apoptosis: genetic controls of cell death", pages 777-779, see entire document.	1-61
Y	CELL, Volume 75, issued October 1993, D.M. Hockenberry et al., "Bcl-2 functions in an antioxidant pathway to prevent apoptosis", pages 241-251, see entire document.	1-61
Y	BIO/TECHNOLOGY, Volume 11, issued 11 July 1993, S.M. Edgington, "Looking death in the eye: Apoptosis and cancer research", pages 787-792, see entire document.	1-61
Y	CELL, Volume 67, issued 29 November 1991, A. Strasser et al., "bcl-2 transgene inhibits T cell death and perturbs self-censorship", pages 889-899, see entire document.	1-61
Y	CELL, Volume 67, issued 29 November 1991, C.L. Sentman et al., "bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes", pages 879-888, see entire document.	1-61
Y	CELL, Volume 47, issued 10 October 1986, M.L. Cleary et al., "Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation", pages 19-28, see entire document.	1-61
Y	IMMUNOLOGY TODAY, Volume 12, number 4, issued 1991, J.C. Ameisen et al., "Cell dysfunction and depletion in AIDS: the programmed cell death hypothesis", pages 102-105, see entire document.	1-61

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A01N 43/04, 63/00; A61K 31/70, 37/00; C07K 16/00, 16/18; C12N 1/08, 1/21, 5/00, 5/06, 5/16, 7/00, 15/09, 15/13

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/93.21, 130.1, 141.1; 435/6, 7.1, 7.2, 7.21, 7.24, 7.7, 7.8, 69.1, 70.1, 240.21, 320.1; 514/2, 44; 530/387.1; 800/2

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Databases: APS, CA, BIOSIS, MEDLINE, EMBASE, DERWENT, USPATFULL, SCISEARCH
Search Terms: apoptos?; bcl?; cdn?; cdi?; protein?; cdna; dna?; antibod?; transgen?; polyclon?; monoclon?; T (w) cell?; lymphocyt?; immun?; northern?; elisa; pcr; polymerase chain reaction; gene; therapy; ex vivo; in vivo; superoxide; dismutase; sod?; yeast

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